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# Studies on Foreign Gene Transfer into Fish

Koji Inoue

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## Abbreviations

BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FLV	Friend leukemia virus
GH	growth hormone
GVBD	germinal vesicle breakdown
HBS	HEPES-buffered saline
LTR	long terminal repeat
MLV	Moloney leukemia virus
mMT-I	mouse metallothionein I
mRNA	messenger RNA
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMS	pregnant mare serum gonadotropin
RNA	ribonucleic acid
RSV	Rous sarcoma virus
rtGH	rainbow trout growth hormone
rtMT-A	rainbow trout metallothionein A
RT-PCR	reverse transcription-PCR
SDS	sodium dodecylsulfate
SV40	Simian Virus 40
TLC	thin-layer chromatography
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside

## Introduction

Explosive progress in genetic engineering in the 1970's made it possible to isolate eukaryotic genes, and is resulting in the accumulation of an enormous amount of information on their structure and function through *in vitro* experiments. The *in vitro* study alone, however, can not be the basis for conclusions, because genes exist and function in living individuals. Early in the 1980's revolutionary technique was developed to introduce isolated genes into living animals and investigate their function *in vivo*. This technique is microinjection of foreign genes into eggs and production of animal lines carrying the injected genes. Individuals carrying such foreign genes are called "transgenic" animals and have been produced in various species including *Caenorhabditis elegans* (Stinchcomb et al. 1985), *Drosophila* (Spradling and Rubin 1982; Rubin and Spradling 1982), sea urchin (McMahon et al. 1984; 1985; Flytzanis et al. 1985; 1987; Colin et al. 1987; Hough-Evans et al. 1987; Katula et al. 1987; Franks et al. 1988a, b; Vitelli et al. 1988), amphibians (Rusconi and Schaffner 1981; Etkin 1982; Bendig and Williams 1983; Etkin and Pearman 1984, Etkin et al. 1987; Wilson et al. 1986), mice (Gordon et al. 1980; Palmiter and Brinster 1986), and farm mammals (Hammer et al. 1985; Brem et al. 1985). This technique has been particularly well established in mice and *Drosophila*, and used as a practical tool for studying genetic processes in such fields as developmental biology, immunology, neurobiology and oncology, and for producing new experimental animals useful in biological and medical research.

Application of transgenic technique to fish began in the middle of 1980's (Maclean et al. 1987), about 5 years later after the first report of transgenic mice (Gordon *et al.* 1980). Fish is the most diversified group of vertebrates. An enormous number of fish species are distributed in a wide variety of environment. Each species has variety of features to adapt to environmental conditions including physical, geographical and ecological factors. Such diversified features of various fish species offer useful models for studying various biological phenomena (Powers 1989). In addition, some species such as medaka (*Oryzias latipes*) and zebrafish (*Brachydanio rerio*) have been recognized as excellent experimental animals in

vertebrates (Yamamoto 1975; Kimmel 1989). Thus development of transgenic systems is expected to contribute to the advance of molecular-biological studies of vertebrates.

Fish is important not only as experimental animals but also as food resources. In recent years, fish culture has received much attention because traditional fisheries depending only on natural populations may cause the decrease in fish resources. Unlike farm animals and plants, however, few special strains for aquaculture has been produced by traditional breeding in spite of the increasing importance of fish culture. Establishment of the foreign gene transfer system in fish is expected to have significant impact upon commercial aquaculture because this technique is expected to become a novel method to produce useful strains for aquaculture.

The first successful introduction of foreign genes into fish was reported in 1986 by two groups. Chourrout *et al.* (1986) microinjected the foreign growth hormone gene into rainbow trout eggs and showed the existence of the transgene in embryos. Ozato *et al.* (1986) microinjected the chicken  $\delta$ -crystallin gene into medaka oocytes and showed the evidence of foreign gene expression. Although these pioneering studies indicated that foreign gene transfer is possible in fish, their systems have not been proved to be entirely successful. The result by Chourrout *et al.* (1986) in which the transgene was detected only by dot blotting was somewhat preliminary and they failed to detect the expression. Ozato *et al.* (1986) clearly showed the expression of the transgene by immunological methods but the tissue specificity of the transgene expression seemed to be lost. In addition, inheritance of transgenes into offspring which is an essential step in transgenic studies has not been examined in these studies.

This study has been performed to establish the system for foreign gene transfer which enable to achieve introduction, expression and germ-line transmission of transgenes in fish. The author established three different methods for foreign gene transfer, microinjection into oocytes, microinjection into fertilized eggs and electroporation, using medaka as an experimental animal. Succeedingly, activities of various promoters derived from fish and other animals in fish cells were estimated using

cultured cells derived from the rainbow trout liver. *In vivo* activities of promoters which were found to be active in fish cells were also examined using medaka embryos. By these experiments, information about the choice of the promoter to achieve appropriate expression of transgenes was obtained. Another objective of this study is to evaluate the potential of transgenic technique as a tool for basic science and genetic engineering. As a model experiment of utilization of transgenic fish in basic studies of genes, the regulation of expression of the chicken  $\delta$ -crystallin gene during the embryonic development was examined using medaka embryos. By this experiment, the potential of the transgenic fish as an effective experimental system to study gene regulation *in vivo* was shown. To evaluate the potential of the transgenic technique as a method for genetic engineering of fish, growth acceleration of rainbow trout (*Oncorhynchus mykiss*) by introducing the growth hormone cDNA linked to a heterologous promoter was also attempted. It was shown that introduced gene was expressed and the growth of injected fry was accelerated. Thus, the transgenic technique became applicable to breeding of economically important species of fish.

## Chapter 1 Development of methods for introducing foreign genes into fish

Medaka (*Oryzias latipes*) is a small egg-laying freshwater teleost which is widely used as a laboratory animal (Yamamoto 1975). It is the only fish species whose several inbred strains have been established (Hyodo-Taguchi and Egami 1985). The oogenesis (Iwamatsu et al. 1988), fertilization (Iwamatsu 1992), and embryonic development (Yamamoto 1975) of this species have been extensively studied. Table 1.1 summarizes biological characters of medaka in relation to transgenic experiments. The 3 cm long size medaka falls into the smallest group of vertebrates known so far. The generation time is short, 2 to 3 months, compared with that of mice. The spawning is daily and the timing of spawning can be controlled by light conditions during a 24-hr period. In addition, the transparency of eggs is distinct advantage for embryological manipulations. Three different methods, microinjection into oocytes, microinjection into fertilized eggs and electroporation each of which has unique features were established by a series of experiments using medaka as a model fish in this chapter.

Table 1.1 Biological characters of medaka

Strain	Orange-red-colored Wild type
Inbred strain	Present
Life span	2 years
Sex maturation	3 months
Total length	3 cm
Spawning	year-round
Spawning cycle	24 h
Days for hatching	8–10 days
Diameter of eggs	1 mm
Transparency of eggs	Transparent
Chorion	Slightly hard
Diameter of oocytes	<1 mm
Diameter of germinal vesicle	100 $\mu$ m
Culture of oocytes	Possible
Chromosome number	48
Genome size	20% of mammals

## 1.1 Microinjection into oocytes

The first method examined was microinjection into oocytes, which has been developed by Ozato et al. (1986). This method was developed to inject DNA solution into the nucleus of eggs. In mice, it is known that foreign genes can be transferred by microinjecting DNA solution into the nucleus of fertilized eggs (Palmiter and Brinster 1986). In fish, however, it is difficult to find nucleus under microscope. On the other hand, the large nucleus (germinal vesicle) is clearly recognized in immature oocytes and thus it is relatively easy to inject DNA solution into it. In this section, the most appropriate stage was determined by injecting plasmids containing rainbow trout growth hormone (rtGH) cDNA into oocytes collected at various stages of maturity. Attempts were also made to improve the medium for oocyte culture. Existence of the transgene in medaka embryos was detected by Southern blotting.

## Materials and methods

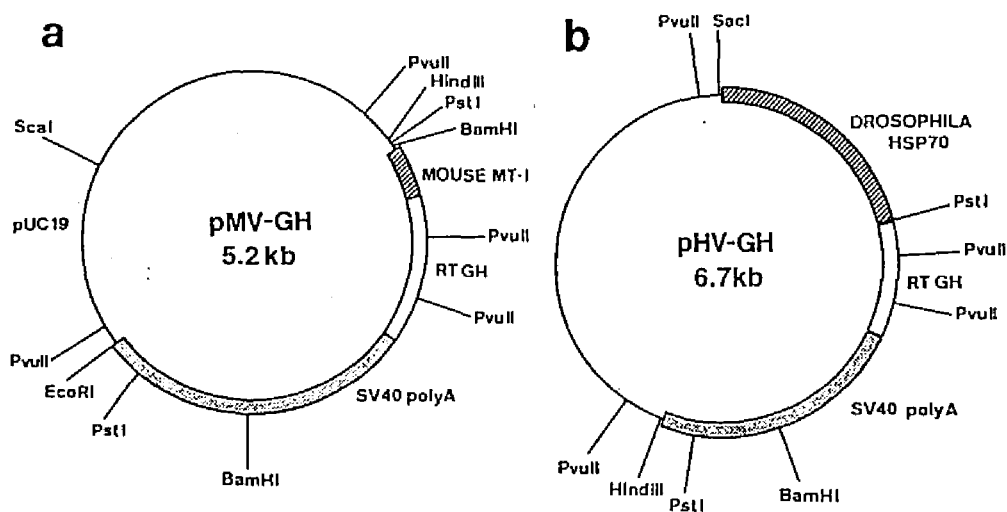
### *Preparation of the plasmid*

The plasmid pMV-GH (Fig. 1.1a) contains the metal-responsive region of mMT-I promoter, rainbow trout growth hormone cDNA and the polyadenylation sequence of SV40. The plasmid pHV-GH (Fig. 1.1b) contains Hsp promoter (Ingolia et al. 1980), rtGH cDNA and polyadenylation sequence of SV40. Plasmids pMV-GH and pHV-GH were linearized with *EcoRI* and *SacI*, respectively, and dissolved in the Dulbecco's phosphate buffered saline (PBS) at the concentration of 10 µg/ml.

### *Microinjection and oocyte culture*

The cultivated orange-red colored strain of medaka was purchased from a commercial source. Experimental fish were bred under controlled photoperiod (14 h light and 10 h dark) at 26 °C. The intrafollicular oocytes were taken out from the ovaries of females from 20 to 6 hours before the anticipated time of ovulation at intervals of 2 h and put into Earle's 199 medium supplemented with 2% bovine serum albumin (BSA),

17.8 mM NaHCO<sub>3</sub>, 25 mg/l penicillin G and 15 mg/l streptomycin sulfate. In some experiments, 50 IU pregnant mare serum gonadotropin (PMS, Teikoku Hormone Mfg. Co.) was added into the medium (Iwamatsu et al. 1974, 1978). About 20 pl DNA solution was microinjected into the oocyte nuclei immediately after the isolation of oocytes. Some of the oocytes were left uninjected as the control. Injected and uninjected oocytes were cultured at 26 °C in the same medium until the anticipated time of ovulation, i. e., the beginning of the light period. Oocytes in which germinal vesicle breakdown (GVBD) were counted as mature oocytes, which were inseminated according to Ozato et al. (1986). Eggs in which perivitellin space was formed were regarded as fertilized. After rinsing in distilled water, fertilized eggs were cultured individually until stage 32 in the Matsui stage (Matsui 1949; Yamamoto 1975) or the hatch-out stage in 96-well culture plates filled with distilled water.



**Fig. 1.1** The structure of pMV-GH (a) and pHV-GH (b). Hatched boxes represent the mouse metallothionein promoter and the *Drosophila* Hsp70 promoter. Open boxes represent the rainbow trout growth hormone cDNA. Dotted boxes represent the SV40 sequence containing the polyadenylation site. Thin lines indicate the plasmid sequences.

### *Detection of foreign sequences*

Genomic DNA was extracted from whole bodies of 7-day-old embryos dechorionized with fine forceps or hatchlings. After incubation in 300 µl lytic buffer containing 50 mM Tris-HCl (pH7.5), 10 mM EDTA, 0.5% SDS, 500 µg/ml Proteinase K at 55 °C for 4 h, samples were extracted once with an equal volume of phenol and then twice with phenol: chloroform: isoamyl alcohol (24: 24: 1). The aqueous phase was then treated with ethanol and the precipitate was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA). The DNA sample for slot blotting was denatured in 0.2 M NaOH for 10 min, neutralized by adding an equal volume of 2 M NH<sub>4</sub>OAc, and immediately fixed onto a nylon membrane. The DNA sample for Southern blotting was digested with PstI, electrophoresed on 0.7% agarose gels, and transferred onto a nylon membrane. Membranes were hybridized with rtGH cDNA labelled with [<sup>32</sup>P]dCTP and exposed to X-ray film.

**Table 1.2** Numbers of oocytes used for microinjection at various stages of maturity

Hours before ovulation	Microinjected oocytes	Control oocytes
20	36	30
18	50	24
16	75	38
14	88	36
12	72	25
10	130	30
8	78	53
6	48	23

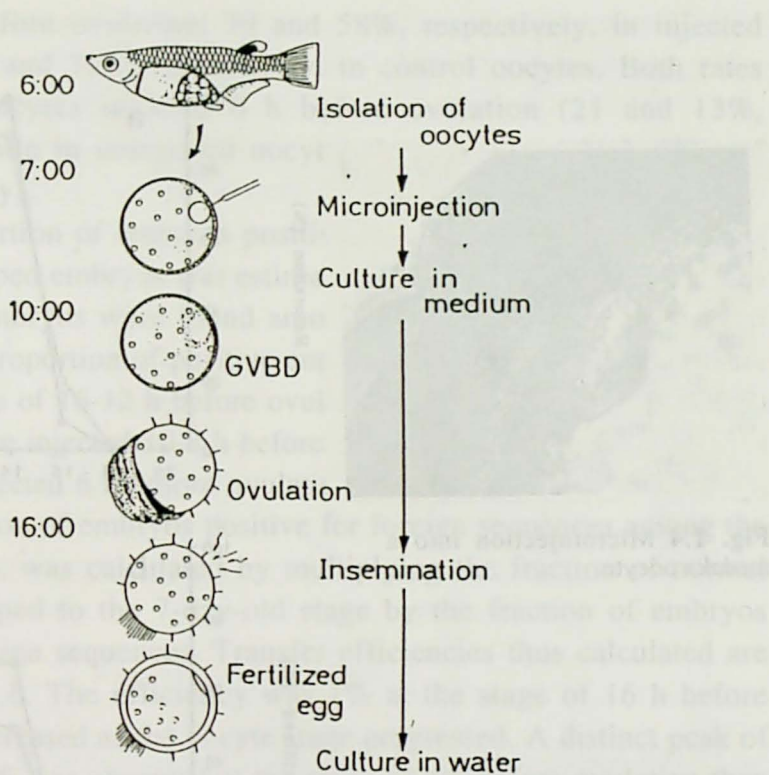
## **Results**

### *Appropriate stages of oocyte maturity for foreign gene transfer*

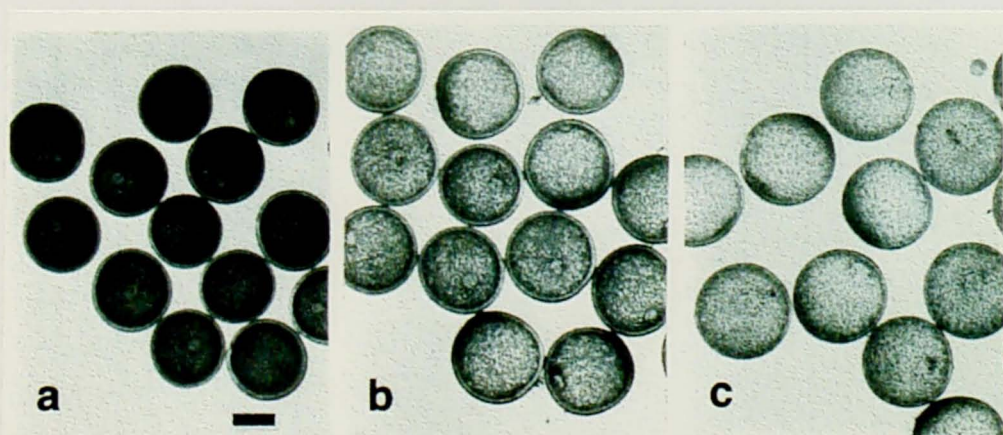
The outline of the procedure of the microinjection into oocytes was described in Fig. 1.2. In the original method, oocytes collected 9 h before



anticipated time of ovulation was used for microinjection. However, it was unknown whether this stage is most efficient for foreign gene transfer. We microinjected DNA solution containing pHV-GH into oocytes collected from 20 to 6 h before ovulation to determine the most efficient stage (Table 1.2) and the the ratios of oocytes matured, fertilized and normally developed to stage 32, after microinjection (Fig. 1.3). Oocytes isolated 20 h before anticipated time of ovulation were approximately 800-900  $\mu\text{m}$  in diameter and were dark in color (Fig. 1.3a). Thus, this stage was classified as stage IX of Iwamatsu et al. (1988). It was not so difficult to microinject the DNA solution into the germinal vesicle at this stage because the germinal vesicle was clearly recognized under a microscope. As oocytes developed, they gradually became larger in size and transparent in color. The size of the oocytes isolated 8 h before ovulation was 1000-1200  $\mu\text{m}$  and the germinal vesicle was also seen clearly near the animal pole (Fig. 1.3b). Thus, microinjection into these oocytes was easier than that into oocytes at earlier stages (Fig. 1.4). The oocytes collected 6 h before ovulation (Fig. 1.3c) had approximately the same size and shape as those of 8 h before ovulation except that GVBD had already begun and the membrane of the germinal vesicle had become fragile in the former. The germinal vesicle disappeared completely in the oocytes isolated less than 6 h before ovulation (GVBD stage). Microinjected and control oocytes were cultured in the medium without gonadotropins. The proportions of oocytes which matured, were fertilized, and developed normally to stage 32 after microinjection are shown in Fig. 1.5. None of the injected control and control oocytes collected 20 and 18 h before ovulation and hardly any of those isolated 16 h before ovulation matured in the medium (Fig. 1.5a). Maturation rates of the oocytes injected 14-8 h before ovulation were 87-90% while those of uninjected oocytes were 88-100% (Fig. 1.5a). The maturation rate remaining high in the uninjected oocytes collected 6 h before ovulation but that of the injected oocytes decreased to 54% (Fig. 1.5a). The proportions of oocytes which were successfully fertilized and developed normally to stage 32 revealed a similar pattern (Fig. 1.5b,c). Both fertilization and development rates were high among oocytes isolated 14-8 h before ovulation, and highest values were obtained at the



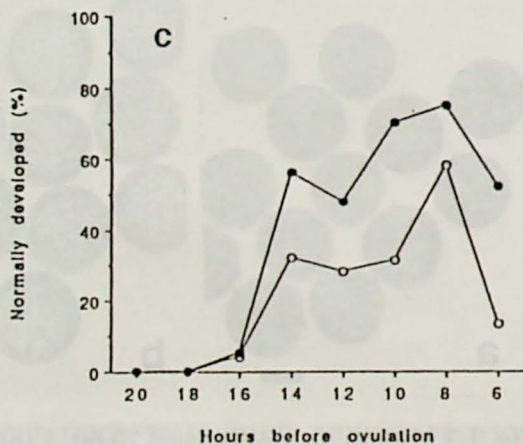
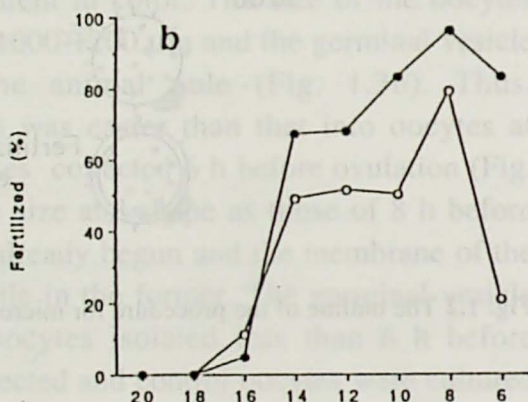
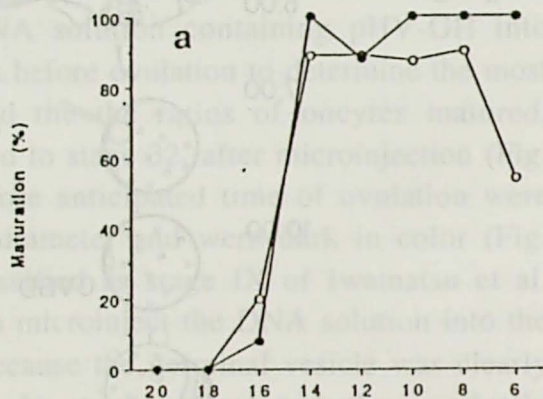
**Fig. 1.2** The outline of the procedure for microinjection into oocytes of medaka.



**Fig. 1.3** Medaka oocytes isolated 20 (a), 8 (b), and 6 (c) hours before anticipated time of ovulation. The bar represents 500  $\mu\text{m}$ .



**Fig. 1.4** Microinjection into a medaka oocyte.



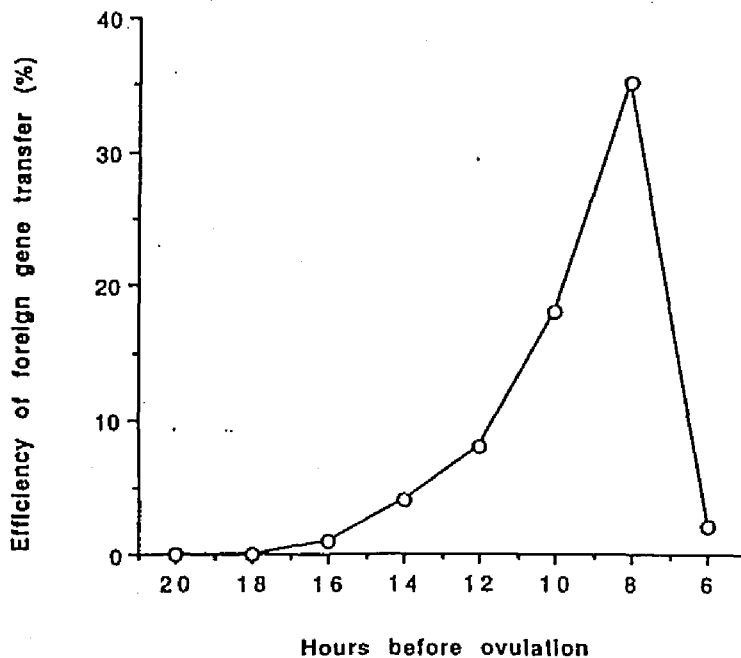
**Fig. 1.5** The ratios of oocytes matured (a), fertilized (b) and developed normally (c) to stage 32, after microinjection. Open and solid circles represent the ratios of microinjected and control oocytes, respectively.

stage of 8 h before ovulation: 79 and 58%, respectively, in injected oocytes and 96 and 75%, respectively, in control oocytes. Both rates decreased in oocytes injected 6 h before ovulation (21 and 13%, respectively) while in uninjected oocytes they remained high (83 and 52%, respectively).

The proportion of embryos positive for foreign sequences among normally developed embryos was estimated using the slot blotting (Table 1.3). Positive embryos were found among those injected 16-6 h before ovulation. The proportion of positive embryos was not high among those injected at stages of 16-12 h before ovulation (13-33%), but increased to 60% among those injected 10-8 h before ovulation and decreased to 17% among those injected 6 h before ovulation. The gene transfer efficiency, i. e. the proportion of embryos positive for foreign sequences among the injected oocytes, was calculated by multiplying the fraction of normal embryos developed to the 7-day-old stage by the fraction of embryos positive for foreign sequences. Transfer efficiencies thus calculated are shown in Fig. 1.6. The efficiency was 1% at the stage of 16 h before ovulation but increased as the oocyte stage progressed. A distinct peak of efficiency at 35 % was observed at the stage of 8 h before ovulation then the efficiency decreased to 2% at the stage of 6 h before ovulation. To sum up, the most efficient stage for foreign gene transfer was 8 h before ovulation.

**Table 1.3** Numbers and proportions of normally developed embryos in which foreign sequences were detected by slot blotting

Stages of oocytes (Hours before ovulation)	Assayed embryos	Positive embryos	%
16	3	1	33
14	15	2	13
12	15	4	27
10	20	12	60
8	20	12	60
6	6	1	17



**Fig. 1.6** Efficiencies of foreign gene transfer by microinjection into oocytes isolated at various stages.

#### *Microinjection into early oocytes*

In the first experiment, gene transfer into oocytes collected more than 16 h before ovulation was impossible because these oocytes lack ability to mature in the medium without gonadotropins. In the second experiment, we attempted to introduce foreign gene into early oocytes using the medium containing a gonadotropin which enhances *in vitro* maturation of oocytes.

Oocytes were collected as described in the first experiment and the DNA solution containing pHV-GH was microinjected. Injected and control oocytes were cultured in the medium containing PMS, inseminated and cultured until stage 32 and assayed for foreign sequences by slot blotting. The result was shown in Table 1.4. Using the modified medium, oocyte maturation was induced even in oocytes collected 20-16 h before ovulation. We obtained fertilized eggs and normal embryos at rates

nearly as high as those found in oocytes collected at later stages (Table 1.4). Embryos which were positive for the foreign sequences were obtained in normal 7-day-old embryos derived from oocytes at every stages, even in embryos from oocytes more than 16 h before ovulation in which no positive embryos was obtained in the first experiment. Only the rate of positive embryos in oocytes collected 8 h before ovulation was lower than in the first experiments.

**Table 1.4** Efficiencies of fertilization, normal development until stage 32, and gene transfer, after microinjection.

Hours before ovulation	Injected				Control		
	No. of oocytes	Fertilized (%)	Normal embryos (%)	Trans-genic (%)	No. of oocytes	Fertilized (%)	Normal embryos (%)
20	16	38	19	6	14	71	50
18	27	78	70	7	15	73	73
16	35	66	51	11	27	78	74
14	25	48	24	16	19	74	74
12	31	68	42	13	27	85	63
10	22	73	36	18	7	100	86
8	31	42	26	10	26	81	65
6	23	43	13	9	16	81	63

### *Introduction of pMV-GH*

The plasmid pMV-GH containing mMT-I promoter and rtGH cDNA was introduced into 420 oocytes collected 8 h before ovulation. Oocytes were cultured in the PMS-free medium and 207 (49%) fertilized eggs were obtained and 104 hatched out. Twenty-two hatchlings were analyzed for the existence of introduced sequences by Southern blotting and positive signal was detected in 12 (55%) hatchlings. As shown in Fig. 1.7, a signal of 2.4 kb corresponding to the *Pst*I fragment of pMV-GH was detected in positive individuals. This result indicates the existence of the introduced sequences without receiving degradation. A signal at higher molecular weight was also observed in both injected and control hatchlings when the wash condition with low-stringency was used (Fig. 1.7). This band is supposed to correspond to the genomic GH gene of the

recipient and disappeared when the wash condition with high stringency was used (data not shown).

## Discussion

In the first experiment of this section, the most effective stage for foreign gene transfer to obtain high gene transfer efficiencies using culture medium with or without the gonadotropin. When the medium without PMS was used, gene transfer efficiency was influenced both by the fraction of normally developed embryos of all embryos after microinjection, and by the proportion of foreign gene-positive embryos among the normally developed individuals. Proportions of normally developed embryos were low for oocytes collected earlier than the stage of 14 h before ovulation using the medium without PMS. This shows that oocytes acquired the ability to mature in the culture medium around the stage of 14 h before ovulation, which is consistent with the fact that the stimulation of oocyte follicles by gonadotropins occurs at this stage (Iwamatsu 1978). Mature oocytes collected 14-8 h before ovulation were fertilized and developed normally to stage 32 in considerably high ratios. During this period, distinct peaks were observed 8 h before ovulation in the fertilization ratio and the normal development ratio. These ratios decreased 6 h before ovulation. These decreases may be due to damages caused by microinjection, because the germinal vesicle was beginning to disappear and the membrane of germinal vesicle became fragile at this stage. As for the proportion of positive embryos which developed normally, it was not high at the stages of 16-12 h before ovulation, but increased to 60% at the stages of 10 and 8 h before ovulation (Table 1.3), although the reason for this increase is unknown. Thus, the highest gene transfer efficiency was obtained at the stage of 8 h before ovulation, in which both the ratios of normally developed oocytes and of positive embryos among normally developed embryos were the highest.

When the medium containing PMS was used, oocytes collected at early stages, 16-20 h before ovulation, became possible to mature in *in vitro* condition. PMS is supposed to be able to stimulate the oocyte follicle as endogenous hormones do. As the result, embryos positive for foreign



sequences were obtained from oocytes collected at all the stages examined. In addition, gene transfer efficiencies using the medium containing PMS was higher than those obtained using the PMS-free medium. However, gene transfer efficiency was not increased in oocytes collected at the stage of 8 h before ovulation in which the highest gene transfer ratio was obtained using the PMS-free medium mainly because of the decrease in normal development ratio. PMS may affect the development of oocytes at this stage. By these results, it was concluded that the medium containing PMS is efficient for introducing foreign genes into early oocytes but PMS is not required for gene transfer into oocytes collected 8 h before ovulation.

1 2 3 4 5 6 7 8 9 10 11 C C P



**Fig. 1.7** Southern blot analysis of embryos microinjected with pMV-GH. DNA purified from embryos were digested with *Pst*I, electrophoresed on 0.7 % agarose gel, transferred onto a nylon membrane and hybridized with the rtGH probe. Lanes 1-11, injected embryos; C, control embryos; P, 10pg plasmid pMV-GH digested with *Pst*I.

Using oocytes collected 8 h before ovulation and PMS-free medium, plasmid pMV-GH was introduced into medaka. The proportion of positive embryos in normal hatchlings was 55%. The gene transfer efficiency, i. e., the proportion of the positive embryos among microinjected oocytes calculated was 27 %. It was also shown by the result of Southern blotting that the transferred gene existed in medaka without any degradation or modification. Thus, it was shown that high gene transfer efficiency is reproducibly obtainable under the condition found in this study.



## 1.2. Microinjection into fertilized eggs

Microinjection into oocyte nuclei was shown to be an effective method for introducing foreign genes into medaka. This method is, however, applicable only to species in which the technique for *in vitro* culture of oocytes has already been established and the oocyte culture has not established in most of fish species. As a more general method, we attempted to introduce transgenes by microinjection into the cytoplasm of fertilized eggs. The microinjection into fertilized eggs has been attempted on other fish species (for review see Maclean 1987) but has never been reported on medaka.

## Materials and Methods

### *Preparation of the plasmid*

The plasmid pHV-GH (Fig. 1.1a) and the plasmid pMV-GH (Fig. 1.1b) were linearized with *SacI* and *EcoRI*, respectively, and dissolved in the Dulbecco's phosphate buffered saline (PBS) at the concentration of 100µg/ml.

### *Egg collection and microinjection*

Medaka was maintained under controlled photoperiod (14 h light and 10 h dark). Females and males were separated one day before experiment using the tank separator (Nisso Kogyo) and kept separately during light period. They are mated at the beginning of the light period. Eggs were spawned within 5 min after mating. Egg clusters were taken from the abdomen of females 20 min after mating and eggs were separated from the cluster by cutting off the attaching filaments with a fine scissors. In some experiments, eggs were kept at 4 °C until microinjection. Then 100-500 pl of DNA solution was microinjected into the germ disk before the first cleavage. Injected eggs were cultured in 96-well microplates filled with distilled water at 26 °C until stage 32 (Yamamoto 1975) or hatch-out.

#### *Detection of foreign sequences*

Genomic DNA was extracted from whole bodies of 7-day-old embryos dechorionized with fine forceps or hatchlings. After incubation in 300  $\mu$ l lytic buffer containing 50 mM Tris-HCl (pH7.5), 10 mM EDTA, 0.5% SDS, 500  $\mu$ g/ml Proteinase K at 55  $^{\circ}$ C for 4 h, samples were extracted once with an equal volume of phenol and then twice with phenol: chloroform: isoamyl alcohol (24: 24: 1). The aqueous phase was then precipitated with ethanol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA). The DNA sample for slot blotting was denatured in 0.2 M NaOH for 10 min, neutralized by adding an equal volume of 2 M  $\text{NH}_4\text{OAc}$ , and immediately fixed onto a nylon membrane. The DNA sample for Southern blotting was digested with *Pst*I, electrophoresed on 0.7% agarose gels, and transferred onto a nylon membrane. Membranes were hybridized with rtGH cDNA labelled with [ $^{32}\text{P}$ ]dCTP and exposed to X-ray film.

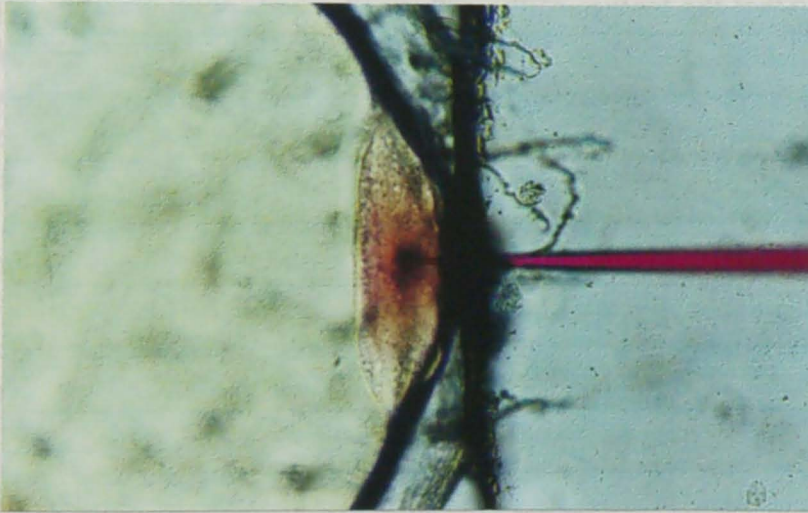


Fig. 1.8 Microinjection into the cytoplasm of medaka fertilized eggs.

## **Results and Discussion**

### *Appropriate stages for foreign gene transfer*

The DNA solution was microinjected into 37 eggs at the early 1-cell stage (stages 1 and 2) and 39 eggs at the late 1-cell stage (stages 3 and

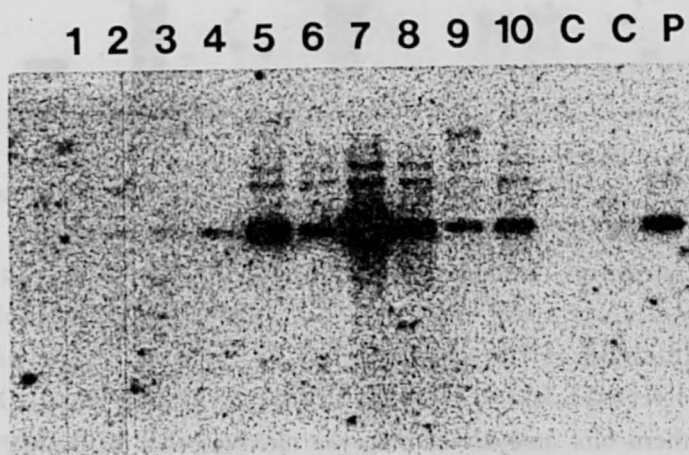
4). The chorion is slightly hard but microinjection was possible using finely sharpened micropipets (Fig. 1.8). Twenty-nine (78%) and 28 (72%) embryos were normally developed to St. 32, respectively. All the normal embryos were assayed for the introduced sequence by slot blotting and 7 (24%) and 7 (25%) embryos were positive (Table 1.5). The gene transfer efficiency was 19 and 18 % for embryos injected at early and late 1-cell stages, respectively. Thus, survival rates and gene transfer efficiencies were almost the same between the two groups. To reduce the rate of generation of the mosaicism, the early 1-cell stage was chosen for further experiments. However, the early 1-cell stage is very short in period and only 30 min is available for microinjection. To elongate the period for microinjection, two methods were tested and both were found to be effective. One is to keep eggs at low temperature to delay the egg development. For example, the development of eggs kept at 4 °C was very slow and remained undivided for several hours. One small problem is that the texture of the cell membrane became flexible as time proceeded and microinjection became slightly difficult during the long storage. The other method is to prepare several aquariums to obtain eggs fertilized at different time. The timing of the spawning can be controlled by the artificial light conditions. In addition, small time differences of spawning were easily produced using the tank separator. Using the separator, it is possible to separate males from females in an aquarium and to mate them at desired time by only removing the separator. We prepared three aquariums and mated females and males each by each at the interval of 30 minutes. Using this procedure, 100-300 eggs can be treated in one experiment.

**Table 1.5** Survival and gene transfer ratios after microinjection into fertilized eggs of medaka at early and late 1-cell stages

Stages	Injected	Developed to St. 32 (%)	Transgenics (% in normal embryos)
Early (St. 1-2)	37	29 (78)	7 (24)
Late (St. 3-4)	39	28 (72)	7 (24)

### Introduction of pMV-GH

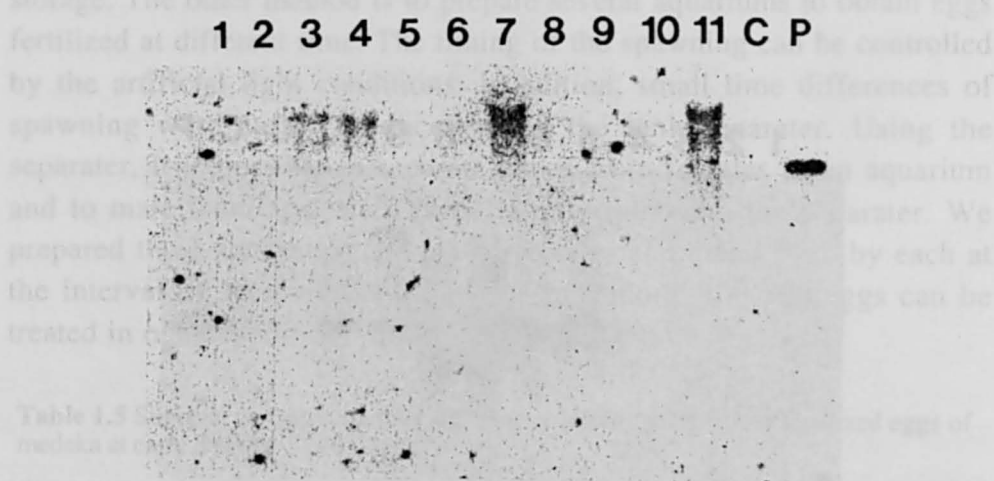
The plasmid pMV-GH was microinjected into 171 fertilized eggs at the early 1-cell stage and 101 (59%) eggs hatched out normally. Total DNA was purified from 21 hatchlings and assayed by Southern blotting. Ten and 11 hatchlings were assayed after the digestion with *Pst*I which cleave the plasmid at two sites and with *Cla*I which has no restriction site on pMV-GH, respectively. Of 10 hatchlings analyzed after digestion with *Pst*I, a 2.4 kb signal which corresponds to the *Pst*I fragment of pMV-GH was detected in 6 hatchlings (Fig. 1.9). This result indicates the foreign plasmid exists in the recipient genome without having been degraded. Of 11 hatchlings assayed after digestion with *Cla*I, a positive signal at high-molecular-weight position was detected in 5 hatchlings (Fig. 1.10). The plasmid is supposed to be integrated into the host genome or at least exists as concatenated DNA. The proportion of positive hatchlings among the assayed hatchlings calculated by combining these results was 52 % and the gene transfer efficiency was 31 %. Thus, the gene transfer efficiency of this method was comparable to that of microinjection into oocytes.



**Fig. 1.9** Southern blot analysis of embryos microinjected with pMV-GH. DNA purified from embryos were digested with *Pst*I, electrophoresed on 0.7 % agarose gel, transferred onto a nylon membrane and hybridized with the rtGH probe. Lanes 1-10, injected embryos; C, control embryos; P, 10pg plasmid pMV-GH digested with *Pst*I.

### *Advantages of microinjection into fertilized eggs*

Microinjection into fertilized eggs has advantages comparing with that into oocytes. The procedure for microinjection into fertilized eggs is more simple than that into oocytes because it does not require oocyte culture, removal of the follicle and insemination. This method is applicable to many fish species for in vitro culture of oocytes has never been established. The efficiency of gene transfer was comparable to microinjection into oocytes despite that DNA solution was not injected into the nucleus. Fertilized eggs are obtainable without killing the parental fish. This point is especially important in experiments using special fish strains such as inbred strains and mutants. Thus, this method is supposed to be more advantageous than microinjection into oocytes. The results obtained here contrast with those in mice in which gene transfer efficiency was very low when foreign DNA was injected into the cytoplasm (Brinster et al. 1985).



**Fig. 1.10** Southern blot analysis of embryos microinjected with pMV-GH. DNA purified from embryos were digested with *Clal*, electrophoresed on 0.7 % agarose gel, transferred onto a nylon membrane and hybridized with the rGH probe. Lanes 1-11, injected embryos; C, control embryos; P, 10pg plasmid pMV-GH linearized with *SacI*.

### 1.3. Electroporation

The microinjection into oocytes and fertilized eggs was proved to be an effective method for introducing foreign genes into fish. The latter is more advantageous in most purposes as described in the previous section. However, even microinjection into fertilized eggs involves certain difficulties. One problem is microinjection itself is a complicated operation under the microscope which requires a great deal of skill. The other problem is the chorion of fertilized eggs. Eggs of many fish species other than medaka are covered with a tough and/or opaque chorion which prevents insertion of the micropipette. To overcome this problem, special methods have been developed in each species, e. g., penetration of micropipetes through the chorion by two-step injection in salmonids (Chourrout et al. 1986; McEvoy et al. 1988); injection through the micropile in tilapia (Brem et al. 1988; Fletcher et al. 1988); dechorionization in goldfish (Zhu et al. 1986), loach (Zhu et al. 1985) and zebrafish (Stuart et al. 1988). To avoid the difficulties accompanying microinjection, we applied electroporation which is often used for gene transfer into culture cells (Neumann et al. 1982; Potter et al. 1984) to the production of transgenic fish.

## Materials and Methods

### *Preparation of the plasmid*

The plasmid pMV-GH (Fig. 1.1a) contains the metal-responsive region of mMT-I promoter, rainbow trout growth hormone cDNA and the polyadenylation sequence of SV40. After linearization with *EcoRI*, the plasmid was dissolved in the mannitol buffer containing 0.25 M mannitol, 0.1 mM  $\text{CaCl}_2$ , 0.1 mM  $\text{MgCl}_2$ , 0.2 mM Tris-HCl (pH 7.5) at the concentration of 100  $\mu\text{g/ml}$ .

### *Egg collection*

Medaka was maintained under controlled photoperiod (14 h light and 10 h dark). Females and males were separated one day before

experiment using the tank separator (Nisso Kogyo) and kept separately during light period. They are mated at the beginning of the light period. Eggs were spawned within 5 min after mating. Egg clusters were taken from the abdomen of females 20 min after mating and eggs were separated from the cluster by cutting off the attaching filaments with a fine scissors.

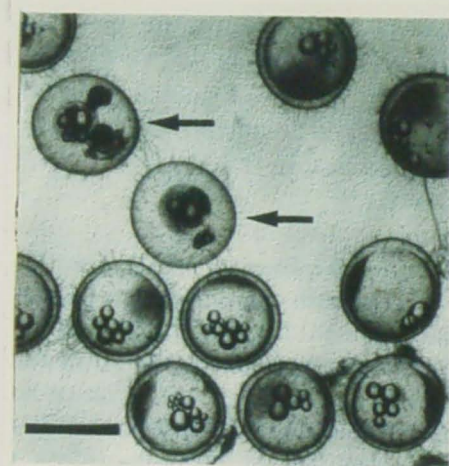
### *Electroporation*

Isolated eggs were put between the electrodes of a transfection chamber, Shimadzu FTC-03 (electrode distance, 2 mm), filled with 800  $\mu$ l of the DNA solution. With this chamber, about 120 medaka eggs could be treated at a time. Before first cleavage, electric pulses were applied by a gene transfer equipment (Shimadzu GTE-1) under the following condition: pulse height, 750 V/cm; pulse interval, 1 s; pulse length, 50  $\mu$ s; pulse number, 5. Treated eggs were rinsed in distilled water several times and incubated separately in 96-well plastic microplates filled with distilled water until hatching at 26 °C.

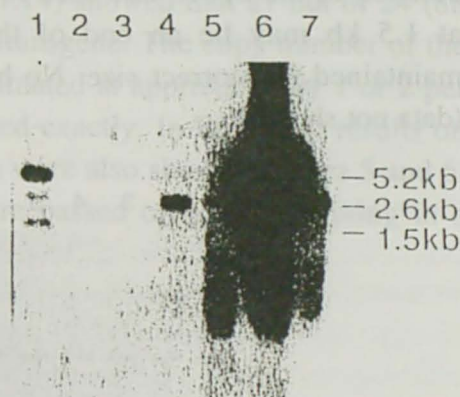
### *Slot blot and Southern analysis*

Genomic DNA was extracted from whole bodies of hatchlings or tail fin pieces of adult fish. After incubation in 300  $\mu$ l lytic buffer containing 50 mM Tris-HCl (pH7.5), 10 mM EDTA, 0.5% SDS, 500  $\mu$ g/ml Proteinase K at 55 °C for 4 h, samples were extracted once with an equal volume of phenol and then twice with phenol: chloroform: isoamyl alcohol (24: 24: 1). The aqueous phase of was then precipitated with ethanol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA). The DNA sample for slot blotting was denatured in 0.2 M NaOH for 10 min, neutralized by adding an equal volume of 2 M  $\text{NH}_4\text{OAc}$ , and immediately fixed onto a nylon membrane. The DNA sample for Southern blotting was digested with PstI or EcoRI and HindIII, electrophoresed on 0.7% agarose gels, and transferred onto a nylon membrane. Membranes were hybridized with rtGH cDNA leveled with [ $^{32}\text{P}$ ]dCTP and exposed to X-ray film.





**Fig. 1.11** Early embryos of medaka at 5 h after the application of electric pulses. Several embryos were damaged by electric pulses (arrows). Bar=10 mm.



**Fig. 1.12** Southern blot analysis of medaka fry hatched from eggs treated with electric pulses. DNA extracted from hatchlings was digested with *Eco*RI and *Hind*III and hybridized with the rtGH probe.

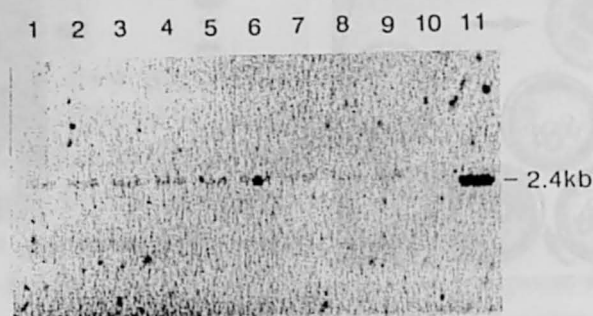
## Results

### *Gene transfer by electroporation*

Of 3109 fertilized eggs treated with pulses, about 10 % died immediately due to damage of the yolk sac (Fig. 1.11). Of the surviving embryos, 783 (25 %) hatched out 8-20 days after fertilization. To examine the efficiency of gene transfer, Southern blot hybridization was performed on 180 hatchlings, and 7 (4% of hatchlings analyzed) were identified as transgenic. A typical result of Southern blotting is shown in Fig. 1.12. In Fig. 1.12, the correct size corresponding to *Eco*RI-*Hind*III fragment of pMV-GH is 2.6 kb. The band at 2.6 kb was observed in all the positive hatchlings. The copy number of the transgene estimated was varied from over 100 (Fig. 1.12, lane 6) to 1 (data not shown). In an individual (Fig. 1.12, lane 1), one major band at 5.2 kb and 2 minor bands at 2.6 kb and 1.5 kb were observed. The size of the major band



corresponds to that of the whole pMV-GH. This may be caused by integration of several copies of transgene in a head-to-tail manner after deletion of the *EcoRI* site used to linearize the plasmid. The minor band at 1.5 kb may be an end of the transgenes. Only the 2.6-kb band maintained the correct size. No band was detected in untreated control (data not shown).



**Fig. 1.13** Southern blot analysis of the F1 offspring obtained from the transgenic founder male and a non-transgenic female. Genomic DNA from F1 fish (lanes 1-9) and a control fish (lane 10) was digested with *PstI*, electrophoresed on 0.7 % agarose gel and hybridized with the rtGH cDNA probe. The plasmid pMV-GH digested with *PstI* was also shown in lane 12.

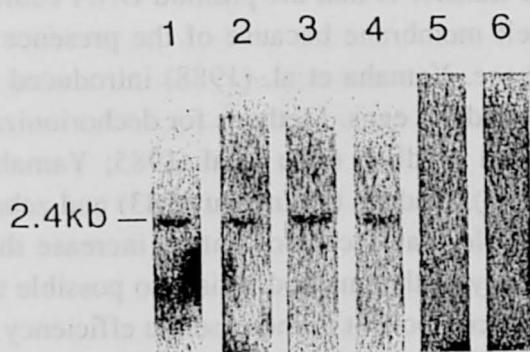
#### *Germ-line transmission of transgene*

To establish transgenic lines bearing pMV-GH, 180 fry hatched from the eggs treated with electric pulses were maintained and 35 matured within 3 months. By slot blot hybridization on DNA extracted from the tail fin of each individual, 2 fish were identified as transgenic (data not shown). One was male and the other was female. Since the female died soon after maturation, we failed to obtain offspring from this fish. From the male, about 100 eggs were obtained by mating normal female. Southern analysis was performed on 17 F1 offspring. Surprisingly, the transgene was detected in all the individuals analyzed (Fig. 1.13). In these F1 offspring, the copy number of transgene was estimated at 1 copy per cell. No band was detected in control fish. (Fig. 1.13, lane 10).

The transgenic F1 offspring also matured within 3 months. By mating a pair of F1 offspring, we could obtain F2 offspring. Southern blot analysis of the F2 offspring (Fig. 1.14) showed that 21 out of 24 (88 %) F2 offspring assayed inherited the transgene. The copy number of the transgene in positive offspring was estimated at approximately 1 or 2 per cell, although it could not be determined exactly. In Fig. 1.14, results on a negative individual and a control fish were also shown in lanes 5 and 6, respectively. Thus, the transgenes were passed on to F2 offspring in a basically Mendelian fashion.

## Discussion

Several methods have been reported for gene transfer into animals. For example, microinjection of DNA, infection with recombinant retroviruses and embryonic stem cell-mediated transfer are now available in mice (Palmiter and Brinster 1986). In this study, we used electroporation as a new method for transferring foreign genes into medaka. Electroporation has often been used for gene transfer into culture cells but not into embryos. This is the first successful application of electroporation to produce transgenic animals.



**Fig. 1.14** Southern blot analysis of the F2 offspring obtained by mating a pair of transgenic F1 offspring. Genomic DNA from F2 fish (lanes 1-5) and a control fish (lane 6) was digested with *Pst*I, electrophoresed on 0.7 % agarose gel and hybridized with the rGH cDNA probe.

**Table 1.6** Comparison of three methods for foreign gene transfer

Methods	Number of eggs treated	Normal embryos survived for 7 days	Frequency of transgenics (%) <sup>a</sup>
Microinjection into oocytes	171	25	55
Microinjection into fertilized eggs	420	59	52
Electroporation	3109	25	4

<sup>a</sup>Frequency of transgenics in normal 7-day-old embryos.

A defect of this method was found to be the low efficiency of gene transfer. The rate of transgenic individuals in hatchlings was only 4 %, far lower than those obtained in microinjection. However, this defect may be overcome by the fact that a large number of eggs can be treated at a time by electroporation. Fish generally spawn enormous number of eggs at one spawning. At this point, electroporation seems especially suited to gene transfer in fish. For practical use, selection markers to distinguish transgenic individuals from non-transgenic ones, such as resistance against antibiotics, should be devised. One of the cause of the low efficiencies in gene transfer is that the plasmid DNA could not come into contact with the cell membrane because of the presence of the chorion and perivitelline space. Yamaha et al. (1988) introduced exotic reagents into dechorionized goldfish eggs. Methods for dechorionization have been already established in goldfish (Zhu et al. 1985; Yamaha et al. 1986), loach (Zhu et al. 1986), medaka (Iwamatsu 1983) and zebrafish (Stuart et al. 1988). It is possible that dechorionization increase the efficiency of gene transfer efficiency in this method. It is also possible to modify pulse conditions and buffer component to increase the efficiency.

In this study, inheritance of the transgene through the germ line demonstrated. This implies that the transgene was stably integrated into host chromosomes. Though germ-line transmission of the transgene is essential for the study of transgenic animals, it has never been demonstrated until this study and Stuart et al. (1988). The inheritance of the transgene by all the F1 offspring was unexpected. This may indicate

that the transgene was integrated into both sets of chromosomes independently although it remains to be proved.

To sum up the results obtained in this chapter, three different methods for introducing foreign genes into fish have been established using medaka as a model fish. Survival rates and gene transfer efficiencies of the three methods were summarized in Table 1.6. Of these methods, electroporation is the most simple, but its gene transfer efficiency is the lowest, although it may be increased by improving the procedure. Microinjection into oocytes is the best with regard to the gene transfer efficiency although it involves complicated procedures and applicable only to limited species. Microinjection into fertilized eggs is less difficult than into oocytes but is obstructed by the chorion in some species. It is important to choose a method for gene transfer according to the purpose of the experiment and the species used.

## **Chapter 2 Estimation of activities of various promoters in fish**

To establish the complete experimental system for transgenic fish, achievement of three steps, introduction, germ-line transmission and expression of transgenes is essential. We have established the methods for introducing transgenes into fish and the transmission of the foreign gene to offspring in the first chapter. The next step to be achieved is the expression of introduced genes. Foreign gene expression of transgenes has never been reported in early studies on transgenic fish except for the report by Ozato et al. (1986) (for review see Maclean et al. 1987). To obtain appropriate expression of transgenes, the choice of regulatory elements is important. A variety of promoters and enhancers derived from genes and viruses of higher vertebrates are available at present and several promoters have also cloned recently from fish. However, information on activities of such regulatory elements in fish is still largely insufficient. In this chapter, activities of various promoters and enhancers in fish were examined in *in vitro* experiments using a fish cell line and in *in vitro* experiments using medaka embryos.

### **2.1. Activities of various promoters in fish culture cells**

For estimating activities of promoters and enhancers, *in vitro* experiments using culture cells are generally performed prior to *in vivo* experiments because the *in vitro* experiments are considered to be more simple than the *in vivo* system. We examined activities of some well-known promoters and enhancers derived from fish and other animals in a series of experiments using a cell line derived from the rainbow trout liver as the recipient of transgenes and the chloramphenicol acetyltransferase (CAT) gene as a reporter.

### **Materials and methods**

#### *Plasmids*

Structures of plasmids used in this section were summarized in Table 2.1. The pSV2CAT is a plasmid containing the promoter-enhancer region of

SV40 in front of the CAT gene (Gorman et al. 1982a). Plasmid pSV0CAT is derived from pSV2CAT by removing the enhancer-promoter (Gorman et al. 1982a). Plasmid pA10CAT was an enhancerless derivative, but possessed a promoter region, of pSV2CAT (Laimins et al. 1982, 1983). Plasmid pAdECAT was constructed by adding the enhancer sequence of adenovirus type 2 to pA10CAT (Hasegawa et al. 1990). Plasmids, pSRCAT, pMLCAT and pFMCFCAT were constructed by inserting the long terminal repeat (LTR) sequences of Rous sarcoma virus (RSV), Moloney murine leukemia virus (MLV) and Friend leukemia virus (FLV) into the *Hind*III site of pSV0CAT, respectively (Hasegawa et al. 1990). Plasmid pmiwCAT was constructed by replacing a part of the 5' upstream region of chicken  $\beta$ -actin sequence of plasmid p $\beta$ act-CAT9 (Fregien and Davidson 1986) with a RSV-LTR sequences containing the promoter and the enhancer regions (Kato et al. 1990). Plasmid pHspCAT was constructed as follows: First, the *Bgl*II-*Pst*I fragment of the 5' flanking sequence of the *Drosophila* Hsp70 gene (Ingolia et al. 1980) was subcloned into the *Bam*HI-*Pst*I site of pUC19 (pUC-Hsp). Secondly, The *Eco*RI site of pUC-Hsp was cleaved and blunt-ended, and *Hind*III linker was added (pUC-H-Hsp). Then the *Drosophila* sequence was isolated using *Hind*III and inserted into the *Hind*III site of pSV0CAT. Plasmid prtMT-CAT was constructed by inserting blunt-ended *Spe*I-*Bam*HI fragment of the rainbow trout metallothionein A (rtMT-A) promoter (Murphy et al. 1990) into the blunt-ended *Hind*III site of pSV0CAT. Plasmid pMV-CAT was constructed as follows: Plasmid pMK containing mouse metallothionein I (mMT-I) promoter (Glanville et al. 1981) was cleaved with *Bgl*II, treated with S1 nuclease and then cleaved with *Bgl*II. This fragment was ligated to the *Bgl*II-*Eco*RI fragment of pSV2CAT containing the terminator region. Resulted *Bgl*II-*Eco*RI fragment was inserted into the *Sma*I-*Eco*RI site of pUC19 (pMV). Then the *Bam*HI fragment of pCM4 containing CAT gene was inserted into the *Bgl*II site at the junction of mMT-I promoter and SV40 terminator, of the pMV.

### *Cell Culture and Transfection*

The cell line RTL-4, derived from the liver of rainbow trout (Watanabe et al. 1987), was used as the recipient. It was grown at 20 °C

in MEM supplemented with 20 % FCS (MEM-20). For transfection,  $5 \times 10^6$  cells were plated in a 100-mm dish and cultured for 2 days. Then the medium was replaced with 4 ml fresh medium and incubated for 4 h. The calcium phosphate-DNA precipitate was prepared by incubating 1500  $\mu$ l HEPES-buffered saline (HBS) (Graham and van der Eb, 1973) containing 20  $\mu$ g plasmid DNA in supercoiled form mixed with 75  $\mu$ l 2.5 M  $\text{CaCl}_2$ , for 15 min. After the addition of the mixture, cells were incubated for 4 h, washed twice with MEM and treated with 15 % glycerol/HBS for 30 s. Cells were then washed with MEM, and cultured in MEM-20 at 20°C for 24 h except the cells transfected with pHspCAT, which were divided into three groups after glycerol treatment and cultured separately at 15, 20 and 25 °C for 48 h.

**Table 2.1** Structures of plasmids used for estimating activities of various promoters and enhancers in the fish cell line, RTL-4.

Plasmid	Promoter	Enhancer	Structure	Terminator	Vector
pSV0CAT	—	—	CAT	SV40	pBR322
pSV2CAT	SV40	SV40	CAT	SV40	pBR322
pA10CAT	SV40	—	CAT	SV40	pBR322
pAdECAT	SV40	AdV	CAT	SV40	pBR322
pSRCAT	RSV	RSV	CAT	SV40	pBR322
pMLCAT	MLV	MLV	CAT	SV40	pBR322
pFMCFCAT	FLV	FLV	CAT	SV40	pBR322
pmiwCAT	Act+RSV	RSV	CAT	TK	pUC18
pHspCAT	Hsp	Hsp	CAT	SV40	pBluescript
pMV-CAT	mMT-I	—	CAT	SV40	pUC19
prrMT-CAT	rrMT-A	—	CAT	SV40	pBR322

Act, chicken  $\beta$ -actin; AdV, human adenovirus type 2; CAT, chloramphenicol acetyltransferase; FLV, Friend leukemis virus; Hsp, *Drosophila* Hsp70; MLV, Moloney leukemia virus; mMT-I, mouse metallothionein I; rrMT-A, rainbow trout metallothionein A; RSV, Rous sarcoma virus; TK, herpes simplex virus thymidine kinase.

### *CAT assays*

10<sup>6</sup> cells were homogenized in 100 µl 250 mM Tris-HCl (pH7.5) and freeze-thawed twice and centrifuged at 12,000xg for 5 min. The supernatant was heated at 60°C for 10 min and centrifuged at 12,000xg for 2 min. Then 56 µl H<sub>2</sub>O and 4 µl [<sup>14</sup>C] chloramphenicol (55 mCi/mmol, 25 µCi/ml) were added to the supernatant and preincubated for 5 min at 37 °C. The preincubated supernatant was mixed with 20 µl 4 mM acetyl coenzyme A and incubated for 16 h at 37°C. The [<sup>14</sup>C] chloramphenicol and its acetylated forms were extracted with 400 µl ethylacetate and separated on silica gel TLC using a solvent system of chloroform: methanol (94:6). TLC plates were dried and exposed to X-ray film for 1 day with an intensifying screen.

## **Results**

### *SV40-related elements*

In the first experiment of this section, activities of a well-known vector pSV2CAT containing SV40 regulatory elements and its derivatives were examined. RTL-4 cells were transfected with pSV2CAT, pSV0CAT, pA10CAT or pAdECAT, and transient CAT activities were assayed. The results are shown in Fig. 2.1. CAT expression was not detected from the cells transfected with pSV0CAT and pA10CAT. On the other hand, strong CAT activity was detected in the cells transfected with pSV2CAT. A considerable level of CAT activity was also detected in the cells transfected with pAdEcat. These results indicate that the SV40 promoter is not expressed alone, but is expressed when it is combined with appropriate enhancers.

### *LTRs of viruses*

In the second experiment, promoter activities of LTRs of Rous sarcoma virus of chicken, Molony leukemia virus and Friend leukemia virus of mouse each of which is known to be active in mammalian cells were examined. Each of the three LTR sequences includes the promoter and enhancer regions of each virus. As shown in Fig. 2.2, the strongest activity was detected upon transfection with pSRCAT. The expression



level of pSRCAT was as high as that of pSV2CAT. pMLCAT was also expressed, although only at a low level. The transfection of pFMCFCAT showed only trace activity.

#### *A chicken chimeric promoter miw*

The activity of the chicken promoter miw (Suemori et al. 1990; Kato et al. 1990) was examined. This promoter consists of the chicken  $\beta$ -actin promoter and the RSV-LTR both of which are expected to be very active in variety types of cells. As shown in Fig. 2.3, pmiwCAT was expressed at a considerable level in RTL-4 although somewhat weaker than pSV2CAT.

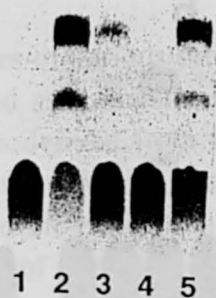
#### *Hsp promoters*

In addition to constitutive elements examined in experiments above, activities of some inducible promoters were also examined. The *Drosophila* Hsp70 promoter is expected to be activated by the heat shock. Cells transfected with pHspCAT were cultured at three different temperatures and assayed for CAT activity. As expected, CAT activity was detected in the cells cultured at 25 °C, a higher temperature than that used normally for culturing, while the cells incubated at 15 and 20 °C exhibited no detectable CAT activity (Fig. 2.4). Thus, the Hsp70 promoter was regulated in a heat-responsive manner in RTL-4.



**Fig. 2.1** Expression of plasmids derived from pSV2CAT in RTL-4. Cells were transfected with plasmids, pSV0CAT (1), pA10CAT (2), pSV2CAT (3) and pAdECAT (4) and assayed for CAT activities.

## Discussion



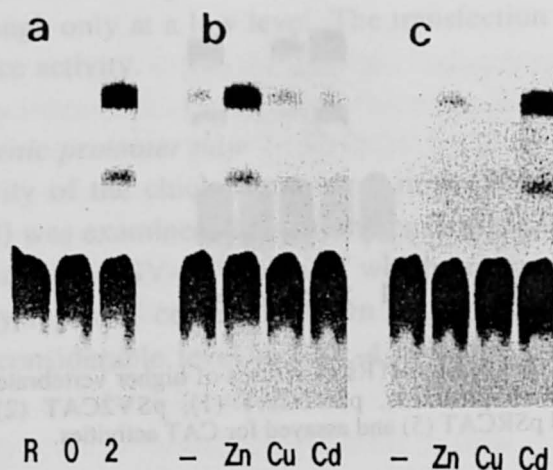
**Fig. 2.2** Expression directed by LTRs of viruses of higher vertebrates in RTL-4. Cells were transfected with plasmids, pSV0CAT (1), pSV2CAT (2), pMLCAT (3), pFMCFCAT (4) and pSRCAT (5) and assayed for CAT activities.



**Fig. 2.3** Expression of pmwCAT in RTL-4. Cells were transfected with pSV0CAT (1), pSV2CAT (2) and pmwCAT (3) and assayed for CAT activities.



**Fig. 2.4** Heat-inducible expression *Drosophila* Hsp70 promoter in RTL-4. Cells were transfected with pHspCAT cultured for 48 h at 15 °C (1), 20 °C (2) and 25 °C (3) and assayed for CAT activities.



**Fig. 2.5** Metal-inducible activities of metallothionein promoters in RTL-4. **a.** controls: Cells without transfection (R) and those transfected with pSV0CAT (0) and pSV2CAT (2). **b.** rtMT-A: Cells transfected with prtMT-CAT and cultured in MEM-20 (–) or same medium containing 100  $\mu$ M zinc chloride (Zn), 100  $\mu$ M copper chloride (Cu) or 100  $\mu$ M cadmium chloride (Cd). **c.** mMT-I: Cells were transfected with pMV-CAT and cultured in the same manner as given in b.

### *Metallothionein promoters*

Metallothionein promoters are expected to be inducible by treatment with heavy metals. To examine activities of mMT-I and rtMT-A promoters, plasmids pMV-CAT and prtMT-CAT were introduced into RTL-4 cells. The results of transfection with prtMT-CAT are shown in Fig. 2.5. CAT activity was low without exposure to metals, but after addition of zinc, expression increased to a level comparable to that of pSV2CAT. Copper and cadmium had no effect. The result of transfection with pMV-CAT are also shown in Fig. 2.5. Only a trace level of activity was observed without induction, but expression was stimulated by adding either zinc or cadmium to the medium. Thus both metallothionein promoters are metal-inducible in RTL-4 cells.

## Discussion

In this section, activities of various promoters and enhancers in the trout cell line were examined as summarized in Table 2.2. Some of the promoters and the enhancers derived from viruses of higher vertebrates exhibited constitutive expression. Among them, the promoter-enhancer of SV40 and RSV-LTR directed the expression at high levels. These results are reasonable because both of them are known as strong promoters in mammalian cells. The activity of the RSV LTR has also been shown in other fish cell lines (Friendenreich and Scharf, 1988). Thus RSV-LTR and SV40 may be used as constitutive regulatory elements to achieve high level expressions of transgenes in fish. The chimeric promoter miw was also shown to be active in RTL-4. The chicken  $\beta$ -actin promoter itself is expected to be active in various types of cells because  $\beta$ -actin is a cytoskeletal actin. In addition, the RSV-LTR which is another component of miw has been shown to be active in this study. Therefore, the result obtained in this experiment is reasonable.

**Table 2.2** Activities of various promoters and enhancers in a trout cell line, RTL-4.

Plasmid	Promoter	Enhancer	Activity	Inducibility
pSV0CAT	—	—	—	—
pA10CAT	SV40	—	—	—
pSV2CAT	SV40	SV40	++	Constitutive
pAdECAT	SV40	AdV	+	Constitutive
pSRCAT	RSV	RSV	++	Constitutive
pMLCAT	MLV	MLV	+	Constitutive
pFMCFCAT	FLV	FLV	—	Constitutive
pmiwCAT	Act+RSV	RSV	++	Constitutive
pHspCAT	Hsp	—	+	Inducible
pMV-CAT	mMT-I	—	+	Inducible
p $\tau$ MT-CAT	$\tau$ MT-A	—	+	Inducible

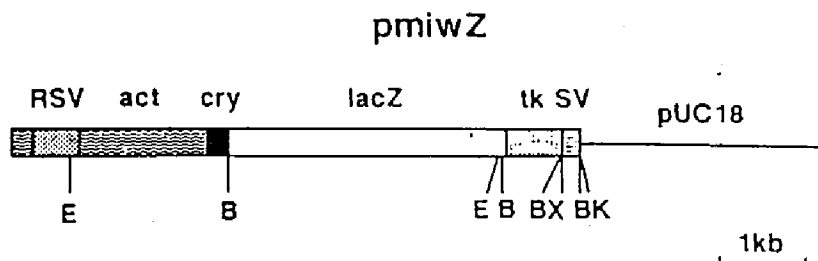
Act, chicken  $\beta$ -actin; AdV, human adenovirus type 2; FLV, Friend leukemia virus; Hsp, *Drosophila* Hsp70; MLV, Moloney leukemia virus; mMT-I, mouse metallothionein I;  $\tau$ MT-A, rainbow trout metallothionein A; RSV, Rous sarcoma virus.

In contrast with these constitutive elements, the *Drosophila* Hsp70 promoter and two metallothionein promoters were expressed in faithful response to heat shock and metal-treatments, respectively. Inducible promoters and enhancers, which can be modulated by environmental factors, are desirable when the transgene products are anticipated to be harmful to host animals. Hsp70 promoter was activated by simple heat-treatment without any harmful substances. Metallothionein promoters is also advantageous in transgenic fish experiment because expression can be induced by adding metals to aquarium water without injection. Therefore, these promoters were supposed to be especially useful in transgenic fish experiments.

Since the activities of promoters are influenced by cell types, the state of differentiation and many other factors, more information is required to elucidate the mechanism of gene regulation in fish. It is yet unknown whether the results of this study are reproducible in *in vivo* experiments. If constitutive and inducible expression systems are established, they will be powerful tools for both genetic engineering of culture fish and basic studies of genes.

## **2.2. Activities of various promoters in fish embryos and fry**

In experiments using the cultured cells, several promoters were found to be active. Among them, activities of two constitutive promoter, the SV2 and the chimeric promoter miw and two inducible promoters, mMT-I and rtMT-A were examined by *in vivo* experiments using medaka embryos to confirm the results obtained in *in vitro* experiments. The activities of the miw and pSV2 were estimated using the  $\beta$ -galactosidase reporter and the CAT reporter, respectively. The activities of the both metallothionein promoters were estimated using the CAT reporter and the induction of the expression was attempted by adding zinc into the aquarium water.



**Fig. 2.6** Structure of pmiwZ. Chicken  $\beta$ -actin sequences (act) including promoter region are indicated by boxes with wavy stripes. RSV LTR sequence is indicated by dotted box. The solid box represents chicken  $\delta$ -crystallin sequence (cry) containing a methionine codon which functions as an initiator codon. The open box represents the bacterial  $\beta$ -galactosidase gene (lacZ). The termination regions derived from herpes virus thymidine kinase gene (tk) and SV40 (SV) are indicated by boxes with vertical and horizontal stripes, respectively. The thin line indicates plasmid vector sequence. Restriction sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; X, *Xba*I.

## Materials and Methods

### Plasmids

The pSV2 CAT is a plasmid containing the promoter-enhancer region of SV40 in front of the CAT gene (Gorman et al. 1982a). Plasmid pSV0CAT is derived from pSV2CAT by removing the enhancer-promoter (Gorman et al. 1982a). Plasmid pmiw Z was constructed by replacing the CAT-coding region of pmiwCAT with the *Sph* I fragment of p $\delta$ Ztk (Ueno et al. 1988) which has a part of the chicken  $\delta$ -crystallin sequence as a translation initiator, the coding sequence of the bacterial  $\beta$ -galactosidase and the termination sequence of herpes simplex virus thymidine kinase gene (Fig. 2.6) Plasmid prtMT-CAT was constructed by inserting blunt-ended *Spe*I-*Bam*HI fragment of the rainbow trout metallothionein A (rtMT-A) promoter (Murphy et al. 1990) into the blunt-ended *Hind*III site of pSV0CAT. Plasmid pMV-CAT was constructed as follows: Plasmid pMK containing mouse metallothionein I (mMT-I) promoter was cleaved with *Bgl*II, treated with S1 nuclease and then cleaved with *Bgl*II. This fragment was ligated to the *Bgl*II-*Eco*RI fragment of pSV2CAT containing the terminator region. Resulted *Bgl*II-

*Eco*RI fragment was inserted into the *Sma*I-*Eco*RI site of pUC19 (pMV). Then the *Bam*HI fragment of pCM4 containing CAT gene was inserted into the *Bgl*II site at the junction of mMT-I promoter and SV40 terminator, of the pMV.

### *Microinjection*

Microinjection into fertilized eggs of medaka was performed as follows: The orange-red type of medaka maintained for more than 4 weeks at 26 °C under controlled light (14 h light and 10 h dark), was used. On the day before microinjection, males were separated from females using the tank separator made of a thin plastic board. On the day of microinjection, males and females were mated by removing the tank separator at the onset of the light. Spawning started within several minutes after mating. Clusters of fertilized eggs were collected from the abdomen of the females 20 min after mating and put into distilled water. Each egg was isolated from egg clusters by cutting the attachment filament. Then about 100 pl of DNA solution was microinjected into the germinal disk before the first cleavage. Injected eggs were washed in distilled water and cultured separately in 96-well microplates at 26 °C until hatching. Embryos were staged according to the Matsui stages (Matsui 1949; Yamamoto 1975).

### *Metal-treatment*

Medaka hatchlings were collected 10 days after fertilization, and maintained in distilled water or exposed to 100 µM ZnCl<sub>2</sub> solution for 6 h. Then hatchlings were rinsed in distilled water and stored at -80 °C until analysis.

### *CAT assays*

Embryos and hatchlings injected with prtMT-CAT or pMV-CAT were homogenized in 100 µl 250 mM Tris-HCl (pH 7.5), disrupted by two cycles of freezing and thawing, and centrifuged at 12,000xg for 5 min. The supernatant was incubated at 60 °C for 10 min and centrifuged at 12,000xg for 2 min. Then, the supernatant was preincubated for 5 min at 37 °C with addition of 56 µl H<sub>2</sub>O, 4 µl [<sup>14</sup>C] chloramphenicol (55

mCi/mmol, 25  $\mu$ Ci/ml), then 20  $\mu$ l 4 mM acetyl coenzyme A was added to the supernatant, and was further incubated for 1 h at 37 °C. [ $^{14}$ C]-chloramphenicol and its acetylated form extracted with 400  $\mu$ l ethylacetate were spotted and separated on silica gel thin-layer chromatography (TLC) plates with chloroform : methanol (94 : 6). The plates were dried and exposed to X-ray film for 1 day with an intensifying screen.

#### *Histochemical staining*

Medaka embryos injected with pmiwZ were fixed with 1.25 % glutaraldehyde in PBS for 3 h and dechorionized with fine forceps. Hatchlings were fixed with the same fixative for 30 min. Fixed embryos and hatchlings were rinsed in PBS and incubated in PBS containing 1.2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), 0.1 % TritonX-100, 1 mM  $MgCl_2$ , 6 mM  $K_4[Fe(CN)_6]$ , 6 mM  $K_3[Fe(CN)_6]$  at 37 °C for 3 h. The embryos and hatchlings were transferred to 1 mM EDTA/PBS to stop the staining reaction.

## **Results**

#### *Activity of pSV2 vector*

The pSV2CAT which was the revealed the highest expression level in RTL-4, was injected into medaka eggs and the expression in hatchlings was examined. The expression was detected in most of hatchlings assayed but the expression level was rather low (Fig. 2.7)

#### *Activity of miw*

The plasmid pmiwZ containing miw promoter and the  $\beta$ -galactosidase reporter microinjected into medaka eggs detected histochemically. Medaka embryos injected with pmiwZ was stained at the gastrula stage (stages 15-17), the 2-day-old stage (stage 24) and the 5-day old stage (stage 29). Expression was detected at all the stages analyzed.



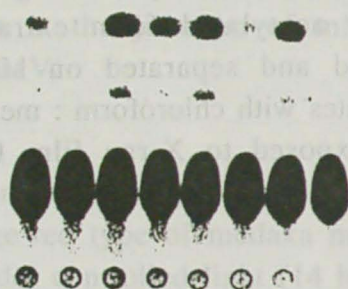


Fig. 2.7 CAT activities of medaka hatchlings bearing pSV2CAT.

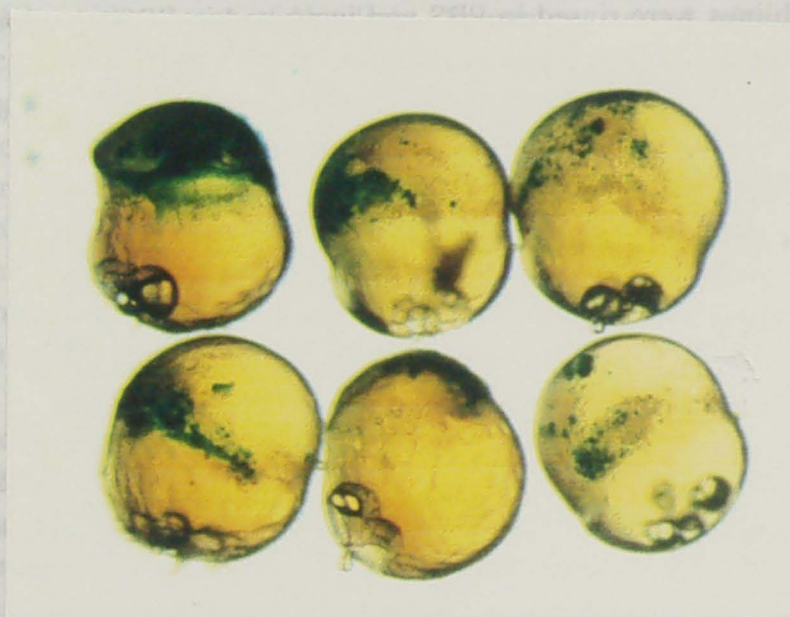


Fig. 2.8 Expression of pmiwZ in medaka embryos at the gastrula stage. Embryos microinjected with pmiwZ were fixed using glutaraldehyde and stained histochemically.

The positively stained cells were mosaically distributed in embryos at the gastrula stage forming patches. However, positive cells were not dominant in each embryo; the rate of the positive cells in embryo were less than 50 % (Fig. 2.8). In 2-day-old embryos, stained cells were distributed in the embryonic body as well as extra embryonic cells but

stained cells were not dominant either (data not shown). As embryos grew, endogenous  $\beta$ -galactosidase activity began to appear. The first organ in which the endogenous activity appeared was the intestine and the distribution of positive cells gradually spread into the yolk. Therefore, it became impossible to detect the expression of the transgene around the yolk and the intestine at later stages. However, the stained cells were found in the muscle where no endogenous activity was detected, in some of the injected 5-day-old embryos (Fig. 2.9). Thus, *miw* was shown to be active in medaka through the embryonic development.

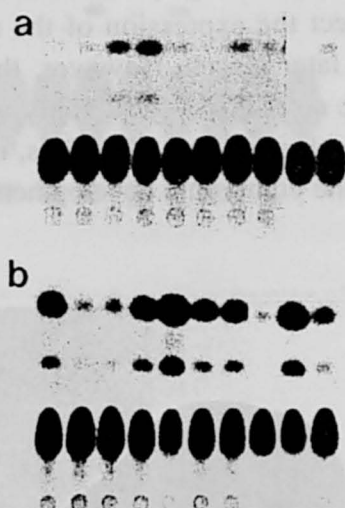


Fig. 2.9 Expression of *pmiwZ* in a medaka embryo at the 5-day-old stage. Embryos microinjected with *pmiwZ* were fixed using glutaraldehyde and stained histochemically.

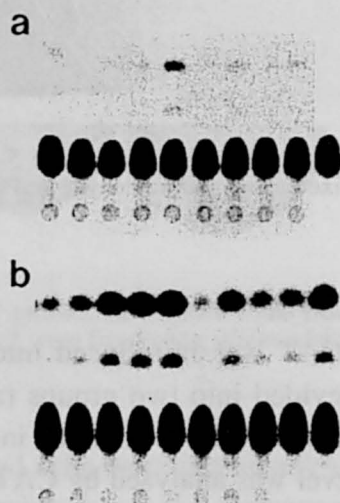
#### *Activities of mMT-I and rtMT-A*

The plasmid *prtMT-CAT* was introduced into medaka eggs. The obtained hatchlings were divided into two groups randomly. One group was exposed to zinc and the other was maintained in distilled water for 6 h and then the expression level was analyzed by CAT assay. As shown in Fig. 2.10., CAT activities were weak in hatchlings maintained in distilled water but was very high in most fry exposed to zinc. Hatchlings injected with *pMV-CAT* were also exposed to zinc or maintained in distilled

water. The results were basically the same as the fry injected with prtMT-A (Fig. 2.11). Thus, it was shown that expression of both rtMT-A and mMT-I was inducible by metal-treatment even in *in vivo* experiment.



**Fig. 2.10** CAT activities of medaka hatchlings bearing pMV-CAT maintained in distilled water (a) and those maintained in 100  $\mu$ M zinc chloride solution (b).



**Fig. 2.11** CAT activities of medaka hatchlings bearing prtMT-CAT maintained in distilled water (a) and those maintained in 100  $\mu$ M zinc chloride solution (b).

## Discussion

In this section, *in vivo* activities of SV2, miw, rtMT-A and mMT-I were examined using medaka embryos. The SV2 is the most popular expression vector in mammal cells. It was also the most active element in RTL-4 cells as shown in the previous section. Thus, the low activity of the SV2 in medaka hatchlings was unexpected. Winkler et al. (1991) reported the expression of SV40 promoter- $\beta$ -galactosidase construct in early medaka embryos. Chong and Vielkind (1989) also reported that pUSV-CAT containing regulatory elements of SV40 and RSV was strongly expressed in early medaka embryos but the expression level decreased as embryos developed. Therefore, such viral elements may be active only in early embryonic stages in fish. In fact, our preliminary experiment showed that pSV2CAT has considerable activity in embryos at stage 22 (data not shown). In contrast to the SV2, miw was shown to be active through the embryonic development. Thus this promoter is a potential promoter for constitutive expression of transgenes. The miw contains the chicken  $\beta$ -actin promoter and the RSV-LTR. The  $\beta$ -actin is a cytoskeletal actin which is expected to be expressed in almost all the cells. The RSV-LTR is also known to have a strong promoter activity in various types of cells (Gorman et al. 1982b) and has also been shown to be active in goldfish and medaka (Yoon et al. 1990; Winkler et al. 1991). Therefore, the strong activity of miw in fish embryos is a reasonable result. The unexpected result, on the other hand, is that the cells expressing  $\beta$ -galactosidase was not dominant in embryos. The reason of the mosaic distribution of expressing cells is unknown at present. At least, it is not due to the incompatibility of the chicken promoters to fish cells because similar results have been reported even in endogenous system in chicken embryos (Naito et al. 1991).

In contrast to these constitutive elements, expression of rtMT-A and mMT-I promoters was inducible by addition of zinc into the aquarium water. At present, rtMT-A is one of the few fish promoters available. Since it has been shown that it is active and inducible in fish fry and cultured cells, this will be an useful expression system in transgenic fish as well as constitutive fish promoters such as carp  $\beta$ -actin promoter (Liu

et al. 1990a,b) and antifreeze protein promoters derived from some cold water species (Du et al. 1992; Gong et al. 1992). It was also found that the mammalian metallothionein promoter functioned well in the metal-inducible manner, and this indicates that the regulation mechanisms for transcription in fish do not differ significantly from those in mammals. This hypothesis is consistent with the fact that sequences of MREs are conserved between fish and mammals (Stuart et al. 1985; Zafarullah et al. 1988; Murphy et al. 1990). The metal-inducibility of both promoters in medaka is clear in contrast to that in transgenic mice in which the mMT-I promoter is used as a constitutive promoter. Especially, rtMT-A promoter faithfully regulated by metal-treatment. Inducible promoter is advantageous for studying function of genes whose expression affect the development of host animals. The constitutive promoter miw and the inducible promoter rtMT-A, which were shown to be active in transgenic fish in this chapter, will be utilized to achieve transgenic expression in future researches.



### **Chapter 3 Stage-dependent expression of the chicken $\delta$ -crystallin gene in transgenic medaka embryos**

Transgenic technique is a powerful tool to study gene regulation *in vivo*. In this chapter, expression of the chicken  $\delta$ -crystallin gene during the embryonic development was examined using the microinjection system established in Chapter 1.

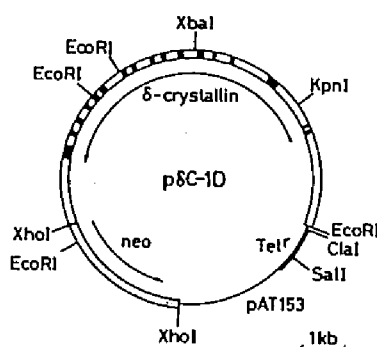
Crystallins are major proteins of the lens of vertebrates and are divided into four major subclasses:  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -crystallins (Clayton 1974; Piatigorsky 1984).  $\delta$ -Crystallin is present in birds and reptiles but in no other vertebrate classes. To study vertebrate crystallin genes, it is of interest to determine the consequence of introducing  $\delta$ -crystallin genes into a vertebrate species which lacks this particular subclass. Extensive studies in which the chicken  $\delta$ -crystallin gene is introduced into mice have shown that the chicken  $\delta$ -crystallin gene is expressed in a tissue-specific manner in cell cultures of mouse embryos (Kondoh et al. 1983; Hayashi et al. 1985), transgenic mice (Kondoh et al. 1987), and chimeric mice (Takahashi et al. 1988). The introduction of the  $\delta$ -crystallin gene into medaka has been attempted by Ozato et al. (1986). They detected expression in 7-day-old embryos but the detailed analysis of developmental regulation was not performed. In this chapter, the  $\delta$ -crystallin gene was again introduced into medaka embryos and expression was examined immunohistologically at several stages of development, from the lens formation stage to one day before hatching. Expression of the gene was observed in the central fiber cells in the lens at the retina-pigmentation stage and in non-lens tissues at stages when the tissues underwent differentiation.

#### **Materials and methods**

##### *Microinjection*

The plasmid p $\delta$ C-1D (17.7 kb, Fig. 3.1) carried the entire  $\delta$ -crystallin gene (Takahashi et al. 1988). This plasmid was dissolved in 100  $\mu$ M Tris-HCl (pH7.5) and 10  $\mu$ M EDTA in circular form at the concentration of 12.5  $\mu$ g/ml. About 30  $\mu$ l of the DNA solution was

microinjected into the germinal vesicle of medaka oocytes 9 h before ovulation. Injected oocytes were cultured in the medium without gonadotropins, inseminated and incubated in distilled water as described in the chapter 1. Embryos were staged according to Matsui stages (Matsui 1949; Yamamoto 1975) Normally developed embryos were sampled at 36 h (stage 22), 60 h (stage 27), 5 days (stage 29) and 7 days (stage 32) after fertilization. Stages 22 and 27 are characterized by lens formation and increasing pigmentation in the retina. The embryonic body encircles the yolk sac entirely at stage 29. Embryos at stage 32 are at one day before hatching.

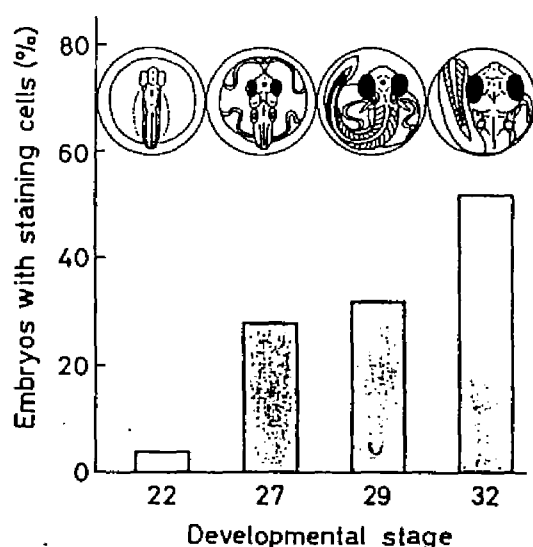


**Fig. 3.1** Structure of the recombinant plasmid, pδC-1D, injected into medaka oocytes. Solid bars represent exon DNA sequences of the chicken  $\delta$ -crystallin gene. Open bars represent flanking and intron DNA sequences of the chicken  $\delta$ -crystallin gene. Dotted bar represents the G418-resistance gene, STneo. Line indicates plasmid vector sequence derived from pAT153 including tetra-cycline resistance gene (Tet<sup>r</sup>). The direction of transcription is indicated by arrows.

### *Immunohistological staining*

Sampled embryos were fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline, embedded in paraffin and sectioned longitudinally at a 5  $\mu$ m thickness. Sections were stained by avidin-biotin-peroxidase complex (ABC) methods according to Hsu et al. (1981). Briefly, sections were deparaffinized, hydrated and treated with 0.3 % H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase activity and with normal goat serum, diluted 1:50 to reduce non-specific staining. Then sections were incubated sequentially with rabbit antiserum against  $\delta$ -

crystallin, diluted 1:200 for 30 min, biotin-labeled anti-rabbit IgG antibody, diluted 1:200 for 30 min, and ABC solution made from 10  $\mu\text{g/ml}$  avidin and 2.5  $\mu\text{g/ml}$  biotin-peroxidase for 1 h. These reagents for ABC method were obtained from Vector Laboratories (Berlingame). Phosphate buffered saline was used for diluting sera and antibodies and washing sections. For coloration, sections were incubated with 0.01 %  $\text{H}_2\text{O}_2$  and 0.05 % diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl (pH7.2), and counterstained with 1 % (w/v) methylgreen. All the staining procedures were carried out at room temperature.



**Fig. 3.2** Percentage of medaka embryos in which the staining cells were detected in at least one tissue by immunohistological staining using rabbit antiserum against  $\delta$ -crystallin.

#### *DNA-DNA in situ hybridization*

*In situ* hybridization using a  $^{32}\text{P}$ -labeled probe was carried out as described by Takahashi et al. (1985), with some modifications. Sections were deparaffinized, hydrated, and dipped in a solution of 0.5 M NaOH and 1.5 M NaCl for 90 s for denaturation of DNA. Then sections were neutralized in 0.5 M Tris-HCl (pH 7.4) containing 1.5 M NaCl and



transferred to 2xSSC (1xSSC is 0.15 M NaCl and 15 mM sodium citrate). After prehybridization with 100 µg/ml denatured salmon DNA for 2 h at 68 °C in 6xSSC containing 0.012% (w/v) heparin, 0.12 % (w/v) Ficoll and 0.12 % (w/v) polyvinylpyrrolidone, sections were hybridized with 70 ng/ml pδC-1D probe in the same solution supplemented by 50 % formamide. The pδC-1D probe was labeled with [<sup>32</sup>P]dCTP by nick translation. Hybridized sections were washed at 50 °C with several changes of 2xSSC for 1 h and with 0.1xSSC for 30 min and dried. Then sections were exposed to X-ray film overnight for autoradiography.

## Results

### *Outline of the expression in whole embryos*

Of 443 microinjected oocytes, 269 (61%) were successfully fertilized and 222 (50%) survived until one day after fertilization. Among those surviving, 181 embryos developed normally until the time of sampling, while 41 were morphologically abnormal. Normally developed embryos, which were injected with the δ-crystallin gene, were sampled and stained immunohistologically at stages 22, 27, 29 and 32. The number of sampled embryos where δ-crystallin was detected in at least one tissue was 1 of 26 (4%), 7 of 25 (28%), 8 of 25 (32%) and 12 of 23 (52%) at stages 22, 27, 29 and 32, respectively (Fig. 3.2). The percentage of δ-crystallin-positive embryos increased markedly with development. Embryos from stage 22, showing minimum number of tissues in which δ-crystallin was detected, were obtained from the same parental group as the embryos from stage 32, which showed the maximum number of them. No δ-crystallin was detected in non-injected embryos.

### *Expression in the lens*

δ-Crystallin in the lens was detected in three embryos at stage 27 (Nos. 27-5, 27-12 and 27-17) and not detected at any other stages (Fig 3.3). δ-Crystallin was localized in the central fiber cells of the lens but not detected in the epithelium (Fig. 3.4). This distribution pattern of the staining cells in the lens was common to these three embryos. The lens at

Developmental stage	Embryo number	Ectoderm				Mesoderm					Endoderm			
		Lens	Brain	Retina	Spinal cord	Gill	Cranial bone	Notochord	Muscle	Kidney	Heart	Gall bladder	Liver	Alimentary canal
22	22-2													
27	27-5													
	27-6													
	27-12													
	27-14													
	27-17													
	27-18													
	27-20													
29	29-2													
	29-5													
	29-10													
	29-11													
	29-15													
	29-16													
	29-17													
	29-23													
32	32-4													
	32-6													
	32-7													
	32-8													
	32-12													
	32-14													
	32-15													
	32-16													
	32-17													
	32-19													
	32-20													
	32-23													

**Fig. 3.3** Tissues in which  $\delta$ -crystallin was detected in each medaka embryo. Black blocks, blocks filled with large dots and blocks filled with small dots indicate the relative level of staining in order, which was estimated by frequency of staining cells in each tissue.

this stage had features distinct from those at stages 29 and 32: many nuclei were observed in the central fiber cells at stage 27, while distribution of nuclei was confined to the epithelium at later stages.

#### *Expression in the non-lens tissues*

$\delta$ -Crystallin was detected for the first time in presumptive muscle cells of the posterior part of the embryonic body of one embryo (No. 22-2) at stage 22 (Figs. 3.3 and 3.5). At later stages,  $\delta$ -crystallin was detected in various types of tissues, which differed from embryo to embryo. At stage 27,  $\delta$ -crystallin was detected in a few mesodermal tissues, such as the notochord, muscle, and gill (Fig. 3.3), though the staining cells were small in number in each tissue. It was noted that all three embryos expressing the exogenous gene in the lens also exhibited expression in the notochord or muscle. At stage 29,  $\delta$ -crystallin was detected in the muscle, notochord, heart, cranial bone, skeleton of the gill, and alimentary canal (Fig. 3.3). Thus,  $\delta$ -crystallin in these two stages was detected mainly in mesodermal tissues, especially in the muscle and notochord. At stage 32, number of staining tissues increased dramatically (Fig. 3.3).  $\delta$ -Crystallin was detected in the brain (Figs. 3.6 and 3.7a), retina (Fig. 3.6), spinal cord (Figs. 3.6 and 3.7b), gill, cranial bones (fig. 3.7c), notochord (Fig. 3.6), muscle (Figs 3.6 and 3.7d), kidney (Figs. 3.6 and 3.7e), gallbladder (Fig. 3.6), liver (Fig. 3.7f) and alimentary canal (Fig. 3.6). In the brain, the staining cells were found in the prosencephalon, mesencephalon, including the optic lobe, and rhombencephalon, including the cerebellum and medulla oblongata. Thus, at stage 32, the central nervous system was one of the major tissues of expression. The staining cells in the gill were confined to the mesodermal part, the skeleton and mesenchymal tissues. In the muscle, the rate of the staining cells to non-staining cells varied from embryo to embryo. For example, the staining cells accounted for about 20 % of all muscle tissues in embryo No. 32-7 (Fig. 3.6) while only one muscle fiber was stained in embryo No. 32-14 (Fig. 3.7d). The increase in the number of  $\delta$ -crystallin-positive embryos at later stages can be ascribed to an increase in the staining tissues of non-lens types.

To sum up these results in the lens and non-lens tissues: i) in the lens,  $\delta$ -crystallin was detected in the central fiber cells at stage 27 in a

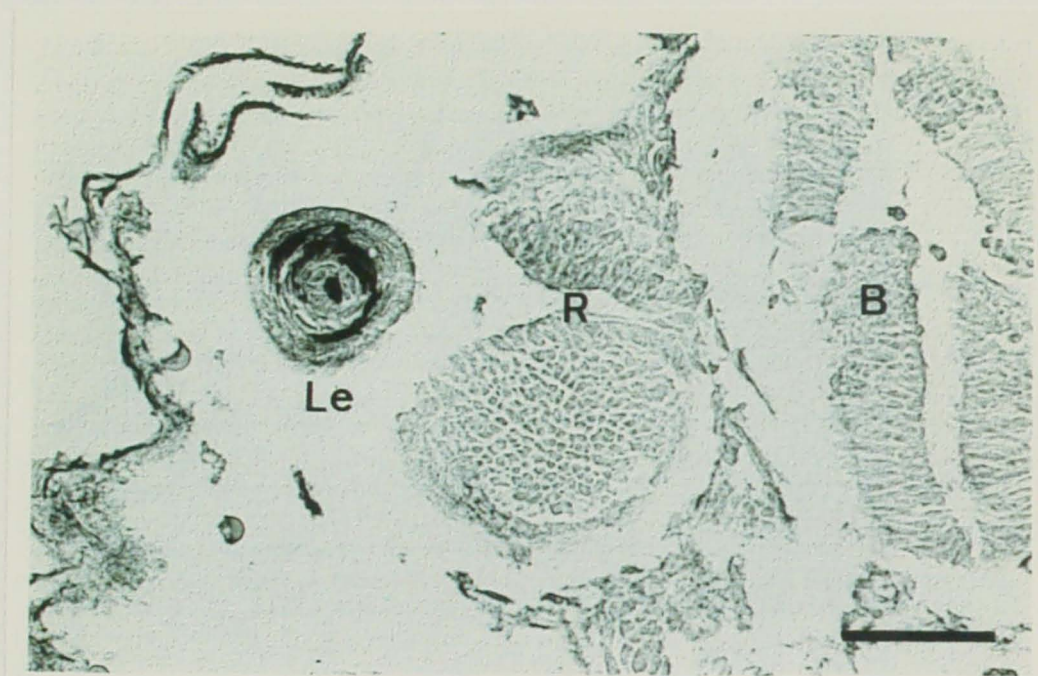
stage dependent manner; ii) in non-lens tissues,  $\delta$ -crystallin was rarely detected at stage 22, while it was detected mainly in mesodermal tissues such as the notochord and muscle at stage 27 and 29, and in many ectodermal and endodermal tissues at stage 32. This indicated that the expression in non-lens tissues was also stage-dependent and the stage of the expression was variable depending on the tissue type.

#### *Expression in abnormal embryos*

Of 41 abnormally developed embryos, 5 and 9 embryos were sampled 5 and 7 days after fertilization, respectively. Thirteen of these 14 embryos developed a dwarfish embryonic body, and 1 formed an undifferentiated cell mass without forming the embryonic body. Three of 13 dwarfish embryos failed to form eye vesicles.  $\delta$ -Crystallin was detected in various tissues of all 14 embryos examined. In the lens, it was detected in 2 of the 5-day-old embryos (Fig. 3.8) and 2 of the 7-day-old embryos. Thus, 4 of 10 abnormal embryos which developed lenses expressed the  $\delta$ -crystallin gene in them. The lenses of these 4 embryos had many nuclei in inner part, as found in the normal embryos at stage 27, but they were not isolated well from the retina; the one exception was a 5-day-old embryo which exhibited normal-shaped lenses, similar to those found in a stage 27 embryo (Fig. 3.8). Abnormal embryos that were not sampled failed to hatch and died within 20 days after fertilization.

#### *Detection of the DNA sequence*

To confirm the existence of the introduced DNA sequence, DNA-DNA in situ hybridization was carried out on several sections of four embryos at stage 32. One was an injected embryo in which  $\delta$ -crystallin was detected (No. 32-7). Two were injected embryos in which  $\delta$ -crystallin was not detected (Nos 32-1 and 32-3) and one was a non-injected control. The DNA sequence was detected in the  $\delta$ -crystallin-positive embryo, but not in the other three. In the former embryo, the injected sequence was distributed throughout the section (Fig 3.9).



**Fig. 3.4** Expression of the chicken  $\delta$ -crystallin gene in the lens of medaka embryo at stage 27 (No. 27-5). B, brain; Le, lens; R, Retina. Bar=50  $\mu$ m.



**Fig. 3.5** Expression of the chicken  $\delta$ -crystallin gene in a medaka embryo at stage 22 (No. 22-2). Arrowhead indicates presumptive muscle cells expressing the  $\delta$ -crystallin gene. Bar=100  $\mu$ m.



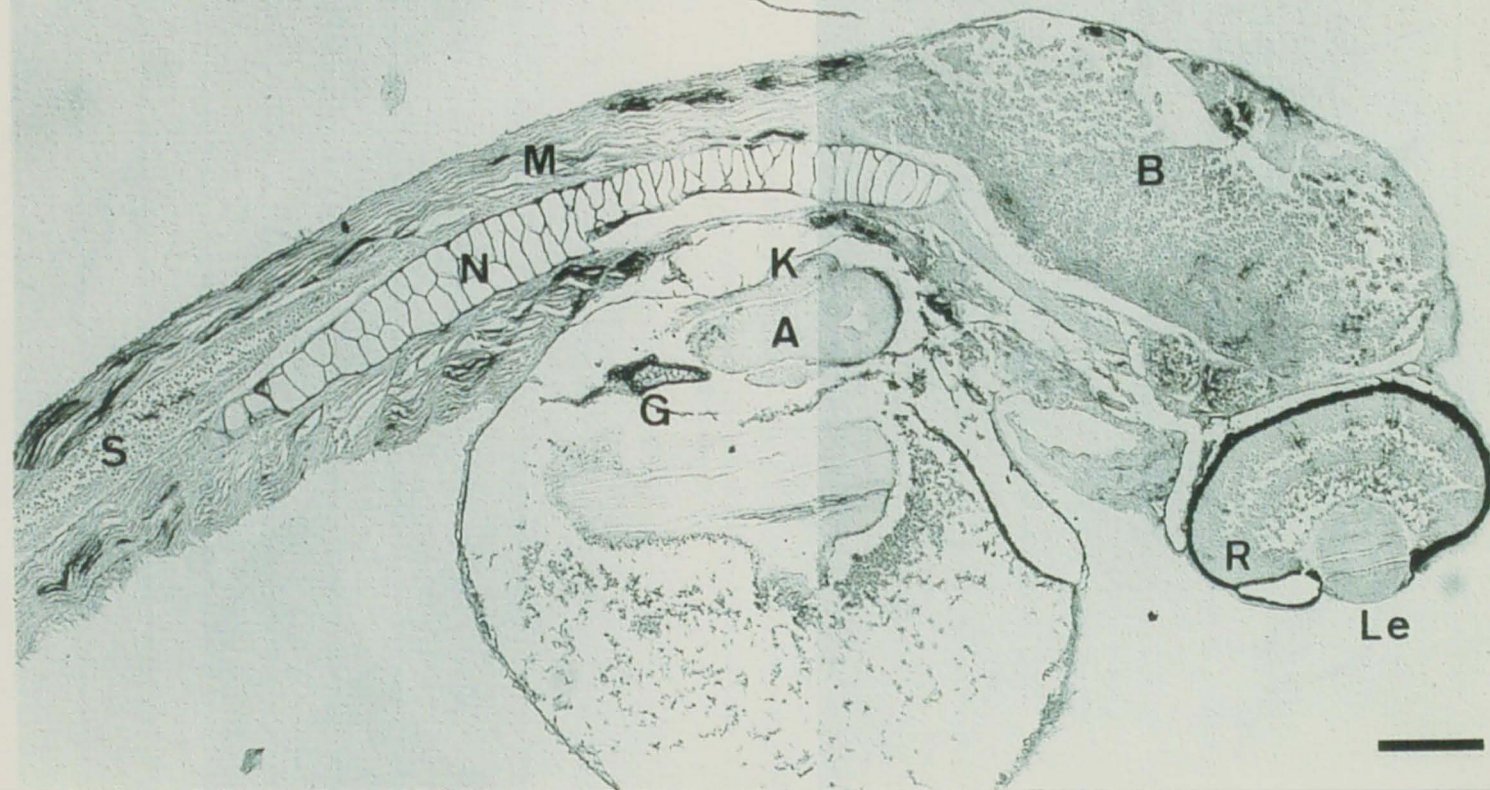
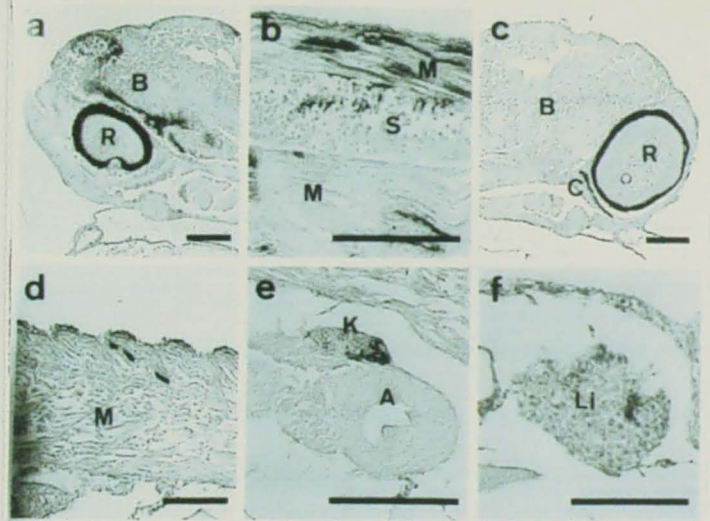
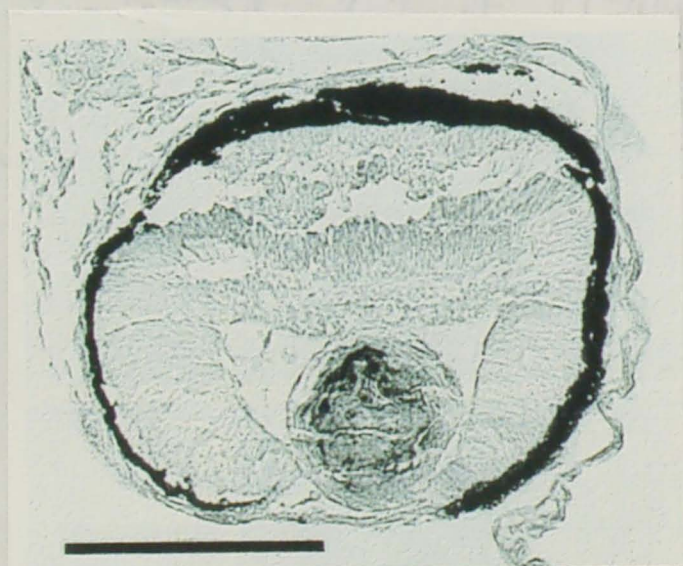


Fig. 3.6 Expression of the chicken  $\delta$ -crystallin gene in a medaka embryo at stage 32. A longitudinal section of the embryonic body of No. 32-7. A, alimentary canal; B, brain; G, gallbladder; K, kidney; Le, lens; M, muscle; N, notochord; R, retina; S, spinal cord. The retina is surrounded by the black pigment epithelium. Bar=100  $\mu$ m.

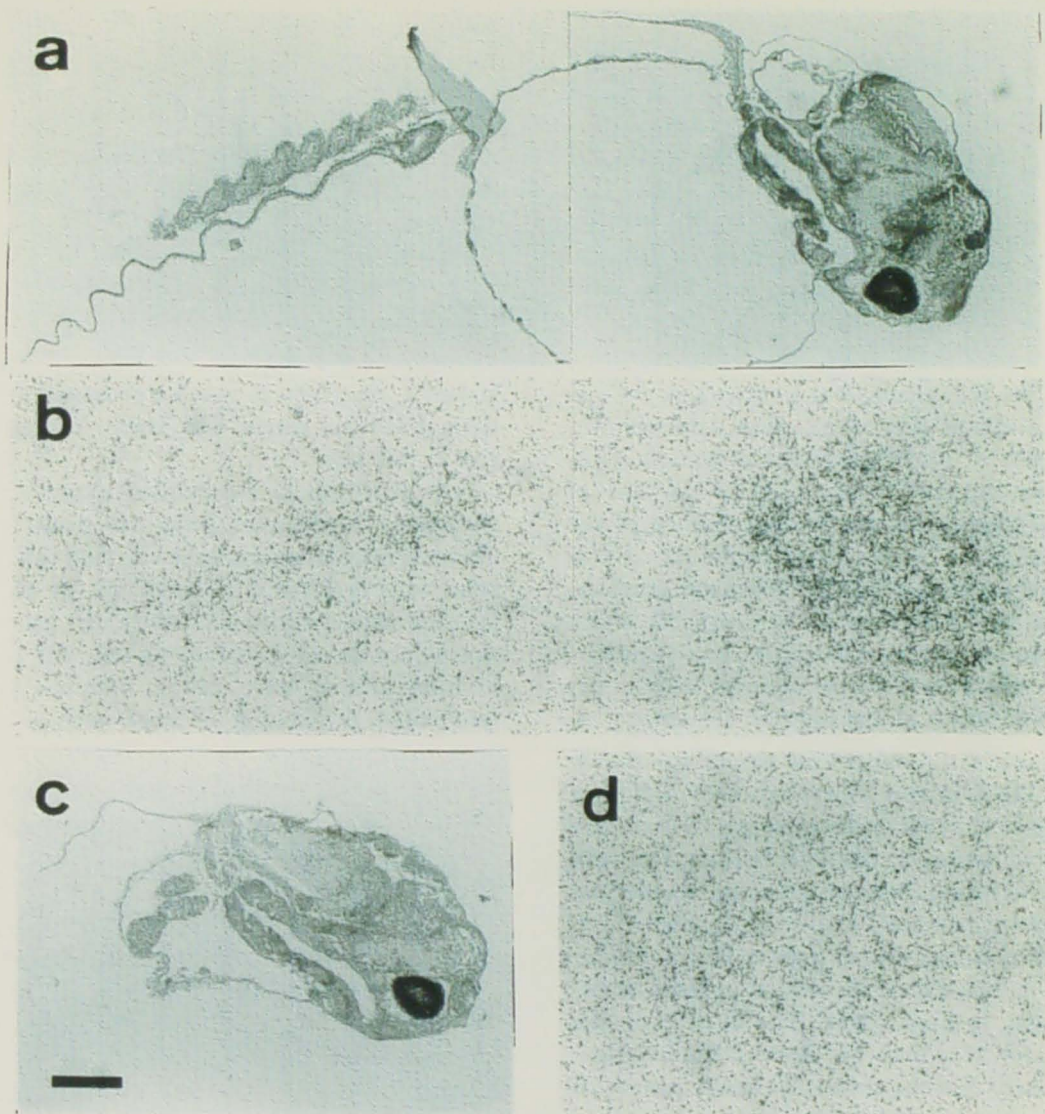


**Fig. 3.7** Expression of the chicken  $\delta$ -crystallin gene in medaka tissues at stage 32. (a) Brain (No. 32-20); (b) spinal cord and muscle (No. 32-7); (c) cranial bone (No. 32-6); (d) muscle (No. 32-14); (e) kidney (No. 32-16); (f) liver (No. 32-15). A, alimentary canal; B, brain; C, cranial bone; K, kidney; Li, liver; M, muscle; R, retina; S, spinal cord. In (a) and (c) the retina is surrounded by the black pigment epithelium. Bar=100  $\mu$ m.



**Fig. 3.8** Expression of the chicken  $\delta$ -crystallin gene in the lens of an abnormally developed medaka embryo. Bar=100  $\mu$ m.





**Fig. 3.9** DNA-DNA *in situ* hybridization with  $^{32}\text{P}$ -labeled p $\delta\text{C}$ -1D probe. (a) A section of a medaka embryo (No. 32-7) at stage 32, stained with hematoxylin. This embryo expressed  $\delta$ -crystallin gene; (b) The autoradiograph of (a); (c) A section of a control medaka embryo at the same stage; (d) The autoradiograph of (c). Bar=100  $\mu\text{m}$ .

## Discussion

In this chapter, expression of the chicken  $\delta$ -crystallin gene introduced into medaka embryos was examined in lens and non-lens



tissues at different stages of development. In the lens,  $\delta$ -crystallin was detected in 3 of 25 embryos at stage 27 but not in embryos at other stages. The protein was also detected in the lens in 4 of 10 abnormal embryos. The lenses of these embryos were morphologically similar to those of a normal embryos at stage 27. It is, therefore, likely that the  $\delta$ -crystallin expression in the lens is stimulated in a stage-dependent manner when the lens reaches a certain state of differentiation. Interestingly, that  $\delta$ -crystallin was localized in the central fiber cells and not detected in the newly differentiating epithelial cells. In the chicken,  $\delta$ -crystallin synthesis occurs mainly in the embryonic lens. With maturation of the lens, synthesis of  $\delta$ -crystallin first decreases in the epithelial cells and subsequently is confined to the central regions of the fiber mass (Piatigorsky, 1981; Tréton et al. 1982). The localized distribution of  $\delta$ -crystallin within the medaka lens may be due to the fact that the  $\delta$ -crystallin expression had occurred in the medaka lens shortly before stage 27 and ceased in the newly differentiating epithelial cells. A similar phenomenon was reported in transgenic mice carrying the  $\gamma 2$ -crystallin gene promoter fused to the coding region of the bacterial  $\beta$ -galactosidase gene (Goring et al. 1987). The enzyme activity was detected in the central nuclear fiber cells of the lens in these mice when they were examined cytochemically at the age of 6 weeks. It is noted that  $\gamma 2$ -crystallin is also an embryonic type of crystallin, similar to  $\delta$ -crystallin. At the present time, it is impossible to make a comparison between the exogenous crystallin gene and endogenous ones, with regard to the developmental pattern of expression in the medaka lens, because there have been no developmental investigations of fish crystallins. Our preliminary observations that crystallin polypeptides were detected from 5 days after fertilization in the normal medaka lens may shed some light on this problem. No  $\delta$ -crystallin was detected in any region of the medaka lens at stages 29 and 32, although it was detected in various other tissues of many embryos. It is assumed that the  $\delta$ -crystallin accumulated in the central fiber cells at earlier stages is decomposed during these stages.  $\delta$ -Crystallin may be unstable in the heterologous lens which naturally lacks it, although it remains in the chicken lens at all stages of development and maturation (Piatigorsky 1984).

The finding that the  $\delta$ -crystallin gene expression in non-lens tissues is stage-dependent, as is found in the lens tissue, was unexpected.  $\delta$ -Crystallin in non-lens tissue was first detected in a small region of undifferentiated mesodermal tissues in one embryo at stage 22. This low level of expression cannot be due to the absence of the exogenous gene, because the specimens at this stage were sampled randomly from the same experimental groups as those at stage 32 which showed the highest level of expression. Similarly, restricted localization of the staining cells is not due to localization of the injected sequences, because the homogenous distribution of the injected sequences throughout the tissues was shown by DNA-DNA in situ hybridization (Fig. 3.9; see also Ozato et al. 1986). Subsequently,  $\delta$ -crystallin was detected mainly in the notochord and muscle, and later in various tissues including ectodermal and endodermal tissues. This stage-dependent expression in non-lens tissues implies that a cellular condition inducing gene expression appears at different stages in each tissue. The most probable condition for this may be tissue differentiation, and it is likely that the stage-dependent pattern of the gene reflects the temporal order in tissue differentiation among the non-lens tissues. Thus, the condition which induces the non-lens type gene expression may rarely be present in undifferentiated tissues at stage 22, while it may occur with progress of tissue differentiation first in mesodermal tissues at stages 27 and 29, and successively in ectodermal and endodermal tissues at stage 32. In fact, mesodermal tissues, such as notochord and muscle, are the earliest tissues undergoing differentiation in medaka embryos (Hiraki and Iwamatsu 1979). However, correlation between  $\delta$ -crystallin expression and tissue differentiation must be confirmed more exactly by further experiments at the cellular level.

The differentiation-dependent expression of the exogenous  $\delta$ -crystallin gene has been found in mouse teratocarcinoma cells carrying this gene. In solid tumors formed by injection of these cells into the mouse peritoneal cavity, the gene expression is not detected as long as the cells are in undifferentiated state, but is detected in a differentiated population of cells such as skeletal muscle cells and secreting epithelial cells (Kondoh et al. 1984; Takahashi et al. 1988). A similar phenomenon has been reported in endodermal cells which differentiated from

teratocarcinoma cells in the presence of retinoic acids (Goto et al. 1988). Thus, the idea that  $\delta$ -crystallin expression in non-lens tissue of medaka embryos depends on differentiation is supported by these experiments on mouse teratocarcinoma cells.

It is an interesting question whether there are any differences in the regulation of  $\delta$ -crystallin expression between the lens and non-lens tissues in medaka embryos. A low level of the  $\delta$ -crystallin expression has been observed in various non-lens tissues of chick embryos, as well as in heterologous experimental systems of mouse carrying this gene. Hayashi et al. (1985) demonstrated, by fine deletion analysis in mouse lens cells and fibroblasts, that the  $\delta$ -crystallin expression could be classified into lens and non-lens types. For instance, dependence of  $\delta$ -crystallin expression on the 5' flanking sequence of the gene differs slightly between lens and non-lens cells of the mouse (see also Kondoh and Okada 1986). An enhancer was identified in an intron of the  $\delta$ -crystallin gene which has both lens-specific and apparently non-specific components (Hayashi et al. 1987). It may thus be reasonable to assume that  $\delta$ -crystallin expression in the lens and non-lens tissues of medaka embryos is due to the activation of lens-specific and non-specific components of the enhancer, respectively, and that the activation of the non-specific components is differentiation-dependent.

In this chapter, stage-specific expression of the chicken  $\delta$ -crystallin gene was elucidated. Results obtained in this experiment indicated that detailed studies on gene regulation is possible using medaka. Among the diversified species of fish, medaka is especially advantageous as a host of foreign gene transfer. This medaka system has the potential of becoming one of the most promising materials for *in vivo* studies of gene regulation in vertebrates.

## **Chapter 4 Application of foreign gene transfer techniques to fish breeding**

The fish cultivation has grown prosperous in recent years. Unlike farm animals and plants, however, few special strains for aquaculture have been developed through traditional breeding because fisheries have largely been dependent upon the natural population for a long time. Efforts are now being made to produce domestic fish strains which have advantageous features for aquaculture using not only traditional means, but also novel methods such as hormone treatment, chromosome manipulation and interspecific hybridization (Donaldson 1986). Techniques for foreign gene transfer are also expected as new methods to breed economically important species. In this chapter, the author attempted to enhance the growth of rainbow trout by introducing growth hormone gene (cDNA) as a model experiment of fish breeding by foreign gene transfer. Rainbow trout is one of the most popular species of fish for aquaculture and is also used as an experimental animal.

### **4.1 The method for introducing foreign genes into rainbow trout**

Eggs of rainbow trout are enough large for embryonic manipulation but are covered with the thick chorion which prevent the insertion of the micro pipettes for microinjection. To penetrate the chorion, micropipets should be more than 15  $\mu\text{m}$  in diameter. To solve this problem, several methods have been reported. Chourrout et al. (1986) devised the "two-step" method. In this method, the micropipets are inserted through the window on the chorion made by thick needles. By this method, foreign genes can be transferred into rainbow trout but it requires much time and skilled technique. Another method has been reported by Yoshizaki et al. (1989): the hardening of chorion is prevented by the glutathione treatment after insemination. It is also possible to penetrate the chorion using micropipets which are more than 10  $\mu\text{m}$  in diameter (direct microinjection). As the first step of the

experiment, foreign gene transfer was attempted using the glutathione methods and its gene transfer efficiency compared with that of the direct microinjection without glutathione treatment.

## **Materials and methods**

### *Preparation of plasmid*

The plasmid pMV-GH (Fig. 1.1) containing the mMT-I promoter, rainbow trout growth hormone cDNA and the polyadenylation sequence of SV40 was linearized with *EcoRI* and dissolved in phosphate-buffered saline at the concentration of 100 µg/ml.

### *Gamete collection, insemination and microinjection*

Trout eggs and milt were collected in January 1989 from 2-year-old fish. About 500 eggs were mixed with 5 ml milt, 300 ml 120 mM NaHCO<sub>3</sub> at 10 °C was added immediately and allowed to stand for 1 min. The eggs were gently rinsed with water several times to remove excess milt and allowed to swell for 3 h at 10 °C in water or in 0.5 mM glutathione (reduced form) the pH of which was adjusted to 10 with 1 N NaOH. After swelling, about 10 nl of DNA solution was microinjected into the cytoplasm of fertilized eggs with micropipets which are about 5 µm or 15 µm in external diameter. Injected eggs and uninjected control were rinsed with water and maintained in circulating water at about 10 °C until hatching.

### *DNA analysis*

Hatchlings were anesthetized with 2-phenoxyethanol. The yolk sac was torn with fine forceps in Dulbecco's phosphate-buffered saline to remove the yolk. Genomic DNA was prepared using Proteinase K-SDS treatment followed by phenol extraction and ethanol precipitation. The existence of foreign DNA was detected using polymerase chain reaction (PCR) as follows: One hundred nanograms of DNA was dissolved in Tth buffer containing 6 µg primer GH7 (5'-GGC TCT TCA ACA TCG CGG TC-3'), 6 µg primer GH8 (5'-TCT TGT TCA GCT GTC TGC GT-3'),

and 200  $\mu$ M dNTP. After preheating at 95 °C for 10 min, 1 unit of *Tth* DNA polymerase (Toyobo) was added and 30 cycles of amplification was performed. Each cycle consisted of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 70 °C. Ten microliters of PCR product was subjected to electrophoresis on 4 % NuSieve 3:1 agarose.

## Results

### *Microinjection without glutathione treatment*

The DNA solution was microinjected using micropipets about 15  $\mu$ m in diameter into the cytoplasm of 100 fertilized eggs of rainbow trout without glutathione treatment and 100 eggs were left uninjected as control. The efficiency of microinjection was not so high because the chorion was too hard to be penetrated even using the thick micropipets. Micropipets were sometimes broken during penetration through the chorion. In addition, precise orientation of eggs was required, otherwise the egg rolled before penetration of the micropipet. Thus, much time, about 4 h, was required to treat 100 eggs. Survival rate of injected eggs was extremely low. Most of the eggs died in early stages of development and only 1 (1%) embryo reached to the hatch-out stage, while 10 (10%) hatched from the control group. The only injected hatchlings was analyzed by PCR but foreign sequence was not detected.

### *Microinjection after glutathione treatment*

The DNA solution was microinjected using micropipets of about 5  $\mu$ m in diameter into the cytoplasm of the first cell of 74 fertilized eggs after the glutathione treatment. A hundred and six eggs were left uninjected as control. Swelling in the glutathione solution prevented the hardening of the chorion effectively and micropipets of about 5  $\mu$ m penetrated the chorion easily. Using this method, 1-3 eggs can be treated in a minute. The hardening of the chorion started again when the glutathione solution was removed by rinse with water. The survival of the injected embryos was not so high, only 5 normal (7 %) hatchlings and 2 abnormal hatchlings were obtained. Abnormal hatchlings had small eye

and bodies. From control eggs, 23 (22 %) hatched out normally. The glutathione solution did not seem to affect the development of embryos at least for the control group. DNA analysis was performed on 5 normal and 2 abnormal hatchlings injected and 2 hatchlings uninjected (Fig. 4.1). The GH7 and GH8 primers used would amplify rtGH cDNA from the portion of +80 to + 193 relative to the point of the translation initiation and generate a 114-bp fragment. In the genomic GH sequence, GH7 and GH8 are located in second and third exons, respectively, and a 138-bp intron exists between them (Agellon et al. 1988a). Therefore, these primers generate a 252-bp fragment when they amplify genomic GH gene. The foreign sequence can be distinguished easily from the genomic GH sequence of the recipient. In Fig. 4.1, the 114-bp band corresponding to the transgene was detected only in the No. 4 hatchling while the 252-bp band corresponding to the genomic GH gene was observed in all the injected and control samples. This evidence indicates that 1 of 5 injected hatchlings was found to have the transgene. Thus, it was shown that foreign gene transfer is possible using the glutathione method.



**Fig. 4.1** DNA analysis of rainbow trout fry microinjected with pMV-GH. Total DNA was extracted from 5 normal injected fry (Lanes 1-5), 2 abnormal injected fry (lanes 6, 7) and control fry (lane 8, 9) were amplified by polymerase chain reaction using primers GH7 and GH8. Amplified products were electrophoresed on a 4 % agarose gel.

## Discussion

In this section, two methods; direct microinjection and the glutathione method was examined. The microinjection into the cytoplasm itself is possible by both methods. However, the results obtained by direct microinjection was quite poor and no positive hatchling was obtained. The glutathione method, on the other hand, worked better; the high survival rate and a positive hatchling was obtained. Although, the gene transfer efficiency was far from satisfaction. The gene transfer efficiency is affected by the survival rate and the proportion of the positive embryos among survived embryos. In this case, it seems that the problem is the survival rate rather than the proportion of positive embryos. Generally the survival of the embryo is largely dependent upon the quality of gametes. And it is said that differences in survival rate between the eggs of high and low qualities are more remarkably recognized after microinjection (G. Yoshizaki, personal communication). Quality of fertilized eggs used in this study did not seem to be so high because the rates of hatch-out in uninjected control were relatively low (10 and 22 % for the two experiments) for rainbow trout which has been adapted to artificial culture conditions. Therefore, the poor rate in this experiment may be due to the egg quality. The quality of eggs are affected by various factors such as the genetic back ground of parental fish and the health but most important factor is the timing to collect the unfertilized eggs. The egg quality is the best just after ovulation and is lost day by day. During the spawning season selection of ovulated females should be carried out frequently. In this experiment, two abnormal hatchlings also appeared. The abnormal phenotype is often caused the insertion of transgenes into the functional genes of not only by the expression products of transgenes but also by the host (Palmiter and Brinster, 1986). In this case, however, transgene was not detected from the DNA of abnormal hatchlings. Dwarfish phenotype is often observed in embryos reared in commercial hatcheries. Thus, it was concluded that these abnormality was not due to the transgene at least in this case although it may be due to the embryonic manipulation with micropipets.



## 4.2. Activity of miw in rainbow trout

Rainbow trout has been often used for transgenic experiments (Chourrout et al. 1986; Rokkones et al. 1989; Guyomard et al. 1989; Penman et al. 1990). However, none of them have achieved the expression except for Rokkones et al. (1989) in which mRNA derived from the exogenous GH gene was detected by northern blot hybridization. The lack of expression is supposed to be due to inappropriate regulatory elements used. The choice of regulatory element to drive the coding region is the most important point to achieve the expression of the introduced gene. In Chapter 2, several active regulatory elements were described in medaka. Among them, the chimeric promoter miw was chosen to drive the GH cDNA in rainbow trout because it is a constitutive element which requires no induction for expression and its activity is expected to continue for longer period than that of the pSV2 vector. Before the use of this promoter to express the growth hormone, its activity in rainbow trout was examined using the  $\beta$ -galactosidase gene as a reporter.

## Materials and methods

### *Gamete collection, insemination and microinjection*

The microinjection experiment was carried out in April, 1990, out of the normal spawning season of rainbow trout of a winter spawning strain in December, 1989 and cryopreserved according to Stoss and Refstie (1983). The collected milt was diluted with 3 ml of the extender containing 0.3 M glucose and 10 % dimethylsulfoxide. The sperm extender suspension was frozen as described by Stoss and Holts (1981). Frozen pellets were stored in liquid nitrogen until insemination. For fertilization, the pellets were dissolved in 120 mM NaHCO<sub>3</sub> solution at 10 °C and immediately added to 350 unfertilized eggs which were obtained from a female trout of a spring spawning strain produced by being kept under the artificially controlled photoperiod. To prevent hardening of the chorion, inseminated eggs were rinsed and swelled 3 h at 10 °C in 0.5

mM glutathione (reduced form) adjusted to pH 10 with 1 N NaOH. The fertilization rate estimated by fixing a part of the inseminated eggs with Bouin's fixative 12 h after fertilization and counting eggs at the cleavage stage, was 80 %. About 10 nl of the DNA solution containing the plasmid pmiwZ (Fig. 2.7) linearized with *Kpn*I (100 µg/ml) in phosphate-buffered saline was microinjected into the cytoplasm of eggs with a micropipet (5 µm in external diameter) attached to a micromanipulator (Narishige Model M-152, Narishige Scientific Instrument Lab., Tokyo Japan) under a dissecting microscope, 3-5 h after fertilization. Injected eggs were rinsed with distilled water and maintained in circulating water at 15 °C.

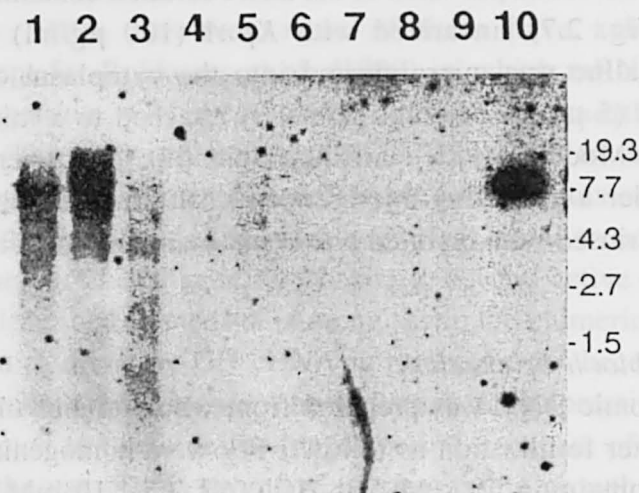
#### *Southern blot hybridization*

Genomic DNA was prepared from whole bodies of individual fry 30 days after fertilization as follows: Fry were homogenized in the lysis solution containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5 % SDS, 500 µg/ml Proteinase K and incubated for 5 h at 55 °C. Then samples were extracted twice with equal volume of saturated phenol and then several times with phenol: chloroform: isoamyl alcohol (24 : 24 : 1). The aqueous phase was precipitated with ethanol and dissolved in TE (10 mM Tris, 1 mM EDTA). Isolated DNA was digested with *Xba*I, electrophoresed on 0.7 % agarose gels, and transferred onto nylon membranes. The DNA on membranes was hybridized with the 3 kb BamHI fragment of pmiwZ containing the coding region of the  $\beta$ -galactosidase gene labeled by random priming with [<sup>32</sup>P] dCTP, and exposed to X-ray film.

#### *Histochemical detection of $\beta$ -galactosidase activity*

Embryos were fixed with 1.25 % glutaraldehyde in PBS for 3 h and dechorionized with fine forceps. Hatchlings were fixed with the same fixative for 30 min. Fixed embryos and hatchlings were rinsed in PBS and incubated in PBS containing 1.2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), 0.1 % TritonX-100, 1 mM MgCl<sub>2</sub>, 6 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 6 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>] at 37 °C for 3 h. The embryos and

hatchlings were transferred to 1 mM EDTA/PBS to stop the staining reaction.



**Fig. 4.2** Southern blot analysis of rainbow trout fry hatched from eggs injected with *pmiwZ*. DNA samples were digested with *Xba*I, separated on a 0.7 % agarose gel, blotted onto a nylon membrane and hybridized with the  $\beta$ -galactosidase probe. Lanes 1–5, injected fry. Lanes 6–9, uninjected control. Lane 10, plasmid *pmiwZ* digested with *Xba*I. The relative migrations of lambda *S*ryI markers are shown on the right.

## Results

### *Introduction of the pmiwZ into trout embryos*

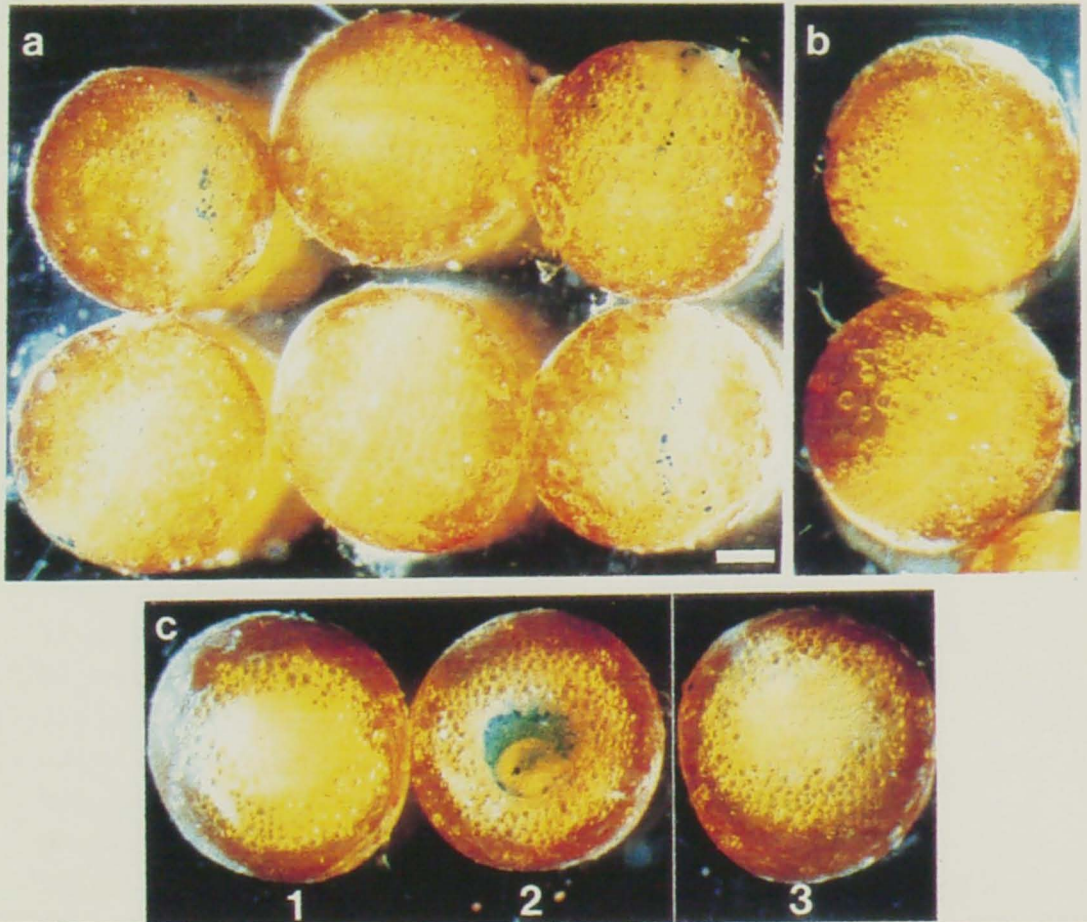
Of 163 eggs injected, 137 (84 %) survived at least until 7 days after fertilization, and 27 (17 %) hatched out 23–25 days after fertilization. Uninjected embryos were also incubated under the same condition. Of 97 eggs, 87 (90 %) survived at least until 7 days and 21 (22 %) hatched out in the same period. The survival rate of the injected embryos was slightly lower than that of the uninjected. This was probably due to the damage caused by the microinjection. The gene transfer efficiency was estimated on 5 fry hatched from injected eggs using the Southern blot hybridization. With the digestion by *Xba*I, foreign sequence was detected at the position of about 9 kb in four individuals (Fig. 4.2, lanes 1, 2, 3

and 5). No signal was detected in uninjected fry. These results indicate the existence of the injected sequences in the each positive fry. However, it is unknown whether the foreign sequences were integrated into the chromosome or not because no junction fragment was detected in positive individuals. The copy number of the transgene were less than one per genome in all the positive fry. This may suggest the mosaic distribution of introduced sequences in each fry. The mosaic distribution of transgene has been reported in previous studies (Ozato et al. 1986; Stuart et al. 1989, 1990; Guyomard et al. 1989).

#### *Histochemical detection of $\beta$ -galactosidase in embryos and fry*

To detect the expression of the  $\beta$ -galactosidase gene, 10 embryos and 10 fry were sampled 7 and 30 days after fertilization, respectively, and stained histochemically. Of the 7-day-old embryos, 6 were morphologically normal, forming the embryonic body.  $\beta$ -Galactosidase activity was detected in all the normal embryos (Fig. 4.3a). Positively stained cells were distributed mosaically in different types of cells in the embryonic body and rarely in the cells on the yolk sac. The frequency and the distribution pattern of the expressing cells were different from embryo to embryo. The other four embryos were morphologically abnormal and failed to form the embryonic body (Fig. 4.3c). Since the similar type of abnormality was frequently observed in control embryos, it is considered that the abnormality was due to the egg quality, but not to the effect of transgenes. Of the four abnormal embryos, one embryo was positively stained (Fig. 4.3c), in which stained cells formed masses in the embryo. No staining was observed in normal and abnormal embryos from uninjected eggs (Fig. 4.3b, c). Thus, positive staining in the embryos at this stage is considered due to the expression of the transgene. Of the 10 fry sampled, 9 were morphologically normal while 1 has a slightly curved body (No. 3 in Fig. 4.4). Upon the histochemical staining, the yolk sac of 3 normal hatchlings (Nos. 1, 2 in Fig. 4.4) and the abnormal hatchling (No. 3 in Fig. 4.4) were intensely stained. No staining was observed in any other organs except the intestine which was weakly stained in both the injected and uninjected hatchlings. This staining

pattern was not changed by the prolonged incubation of fry in the staining solution. The staining in the yolk sac was not detected in control hatchlings. Thus, the specific staining in the yolk sac is considered to result from the expression of the transgene.



**Fig. 4.3** Expression of *pmiwZ* in rainbow trout embryos 7 days after fertilization. Embryos were fixed, dechorionized and stained histochemically for  $\beta$ -galactosidase activity. (a) normal embryos injected with *pmiwZ*. (b) Uninjected normal embryos. (c) Abnormal embryos which failed to form the embryonic body. 1 and 2, injected embryos. 3, an uninjected embryo. Bar=1 mm.





**Fig. 4.4** Expression of pmiwZ in rainbow trout hatchlings 30 days after fertilization. Hatchlings were fixed and stained histochemically for  $\beta$ -galactosidase activity. 1–4, Injected hatchlings. 5–8, Uninjected hatchlings. Bar=5 mm.

## Discussion

The promoter miw (Suemori et al. 1990; Kato et al. 1990) consists of RSV LTR which has a strong promoter activity in various types of mammalian cells (Gorman et al, 1982b), and the chicken  $\beta$ -actin promoter which exhibits a strong activity after stable chromosomal integration (Fregien and Davidson 1986). Thus, this promoter is expected to have a strong and stable activity regardless of host cell types. In fact, nearly ubiquitous expression has been achieved in mouse ES cells and chimeric embryos (Suemori et al. 1990), and it is used in cell lineage analysis in chimeric mice (Kadokawa et al. 1990). It is of interest to see whether miw has a strong promoter activity in fish and is useful for transgenic fish research. The activities of this promoter in fish cells and medaka were already shown in the pervious chapter. In this section, it was shown that miw exhibited a promoter activity in rainbow trout cells, embryos, and hatchlings.

To analyze the *in vivo* activity of miw in rainbow trout, we used bacterial  $\beta$ -galactosidase gene as a reporter. The  $\beta$ -galactosidase reporter is widely used in studies on developmental gene regulation, because the expression can be visualized by histochemical staining. The histochemical method has been used mainly in mouse and *Drosophila* embryos which is relatively small in size. Though the rainbow trout embryos and hatchlings are far larger in size, they were effectively stained. The endogenous  $\beta$ -galactosidase activity was not so high to give a serious obstacle for analysis of gene expression. Thus, the  $\beta$ -galactosidase gene is also useful in trout embryos and hatchlings. This reporter is considered to be a powerful tool in characterizing various promoter in fish.

The microinjection of pmiwZ was carried out in April, out of the normal spawning season of rainbow trout in Japan. The limited spawning season is a serious obstacle for gene transfer experiments. To overcome this, we used cryopreserved milt in the present study. The technique for the cryopreservation of the salmonid milt has already been established (Stoss and Refstie 1983). On the other hand, since it is impossible to store eggs by freezing at present, we used eggs obtained from a female trout matured under the artificially controlled photoperiod. Induction of the maturation by an artificial photoperiod is commonly carried out in Japan and other countries. Using these methods, it became possible to obtain fertilized eggs out of the spawning season. The problem is the quality of the fertilized eggs. Survival rates of the eggs obtained using such methods are sometimes lower than those of eggs naturally obtained. In fact, the hatchling rate of both injected and uninjected embryos in this study was not high, 17 % and 22 %, respectively.

After microinjection, embryos and hatchlings were sampled 7 and 30 days after fertilization, respectively. In 7-day-old embryos, miw showed a strong promoter activity. The staining which showed  $\beta$ -galactosidase activity was detected in various types of cells, almost ubiquitously. The stained cells were distributed mosaically. Although the mosaic distribution of the introduced sequences in transgenic hatchlings was suggested by the fact that the copy number of the transgene in the positive individuals was less than one per genome as shown in Fig. 4.2, it is unknown whether the expression patterns in the present study reflects

distribution of the transgene or difference in its expression among different cells. In hatchlings, the expression was detected in the yolk sac and not in any other tissues. Since miw is constructed from the promoters and an enhancer which are active regardless of host cell types, the tissue specific expression was unexpected. Similar phenomenon has been also in a homologous system, i. e., the transgenic chicken system (Naito et al. 1991). Although the reason of the tissue specificity is unknown at present, the promoter activity of miw was shown in rainbow trout in this section. This promoter will contribute to gene transfer studies as one of the promoters for the expression of genes of interest.

#### **4.3. Introduction of miw-rtGH cDNA construct into rainbow trout**

The rate at which fish grow from egg to market size is an important component of the economics of commercial aquaculture. Especially the growth rate in first several months after hatch-out is a key factor in the successful culture of fish. During this period, fish hatchlings generally complete absorption of the yolk, start to swim and feed. This also a susceptible period to infectious diseases (Wolf, 1988). Thus, shortening of this sensitive phase of the fish life-cycle would be economically beneficial. Administration of growth hormone into fry is a possible method for the purpose. Growth hormone cDNA has been isolated from several fish species and expressed in bacteria (Sekine et al. 1985, Agellon and Chen 1986 Sato et al. 1988, Saito et al. 1988, Hew et al. 1989, Rentier-Delrue et al. 1989, Kosugi et al. 1992). However, it is difficult to administrate growth hormone into hatch-out fry, which are sensitive to the handling.

Transgenic technique is a potential method to deliver growth hormone to fish in commercial culture condition as shown in mice (Palmiter et al. 1982). Using this technique, it is expected that growth hormone can be administrated to fish fry without any damages during handling. As a model experiment to accelerate the growth of fish fry using foreign growth hormone gene, we introduced rainbow trout growth

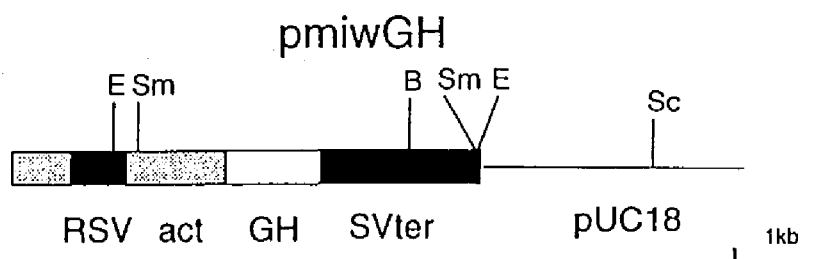


hormone (rtGH) cDNA linked to the promoter miw which was shown to be active in rainbow trout in the previous section, into fertilized eggs of rainbow trout by microinjection. Expression of growth hormone mRNA derived from the foreign plasmid in hatchlings was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). The weight of fry was monitored until 149 days after hatch-out following the microinjection to examine the growth-enhancing effect of the foreign gene product

## Materials and methods

### *preparation of plasmid*

Plasmid pmiwGH (Fig. 4.5) was constructed as follows: The CAT-coding region of plasmid pmiwCAT (Kato et al. 1990) was removed as a 1.5-kb *HindIII-HpaI* fragment and replaced with the *EcoRI* fragment containing rtGH cDNA. A part of the noncoding region of rtGH cDNA and the short SV40 sequence were removed as a *HindIII-BamHI* fragment and replaced with the *BglIII-EcoRI* fragment of pSV2CAT including the termination sequence of SV40. Finally plasmid pmiwGH was dissolved in TE (10 mM Tris and 1 mM EDTA) at a concentration of 100 µg/ml and used for microinjection.



**Fig. 4.5** Structure of the plasmid pmiwGH (7.5kb). Chicken  $\beta$ -actin sequence (act) including the promoter region are indicated by boxes with black dots. RSV-LTR sequence is indicated by the black box with white dots. The open box represents the rainbow trout growth hormone cDNA (GH). The solid box indicates the SV40 sequence including termination region (SVter). The thin line indicates the vector sequence. Restriction sites: B, *BamHI*; E, *EcoRI*; Sc, *ScaI*; Sm, *SmaI*.

### *Gamete collection, insemination, and microinjection*

Rainbow trout eggs and kilt were collected in January 1993 from 2-year-old fish. After about 400 eggs were mixed with 5 ml of milt, 300 ml 120 mM  $\text{NaHCO}_3$  at 12 °C was added immediately and allowed to stand for 1 min. The eggs were gently rinsed several times with water at 12 °C to remove excess milt and allowed to swell for 3 h at 12 °C in 0.5 mM glutathione (reduced form) the pH of which was adjusted to 10 with 1 N NaOH to prevent hardening of the chorion (Yoshizaki et al. 1991). After swelling in the glutathione solution, about 10 nl of DNA solution was microinjected into the cytoplasm of fertilized eggs with a micropipet. Injected and control eggs were rinsed with distilled water to remove glutathione and maintained in circulating water at about 12 °C until hatching.

### *DNA and RNA analysis*

A part of the trout hatchlings was sampled 32 days after fertilization and anesthetized with 2-phenoxyethanol. The yolk sac was torn with fine forceps in sterilized Dulbecco's phosphate buffered saline (PBS) to remove the yolk. Then, each hatchling was divided into two parts: the head part, anterior to the operculum, and the posterior part. The former was used for DNA analysis and the latter for RNA analysis. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until analyzed.

Genomic DNA was prepared using proteinase K-SDS treatment followed by phenol extraction and ethanol precipitation. The existence of foreign nucleic acid sequences was detected using PCR as follows: One hundred nanograms of DNA was dissolved in 1 x Tth buffer containing 6 µg primer GH7 (5'-GGC TCT TCA ACA TCG CGG TC-3'), 6 µg primer GH8 (5'-TCT TGT TCA GCT GTC TGC GT-3'), and 200 µM dNTP. After preheating at 95 °C for 10 min, 1 unit of *Tth* DNA polymerase (Toyobo) was added and 30 cycles of amplification was performed. Each cycle consisted of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 70 °C. Ten microliters of PCR product was subjected to electrophoresis on 4 % NuSieve 3:1 agarose (FMC), transferred to a nylon filter, and hybridized with an oligonucleotide probe (5'-CAG AAA ATG TTC AAT

GAC TTT-3') labeled with [<sup>32</sup>P]ATP. Then the filter was exposed to x-ray film.

Total RNA was isolated using Total RNA Separator (Clontech Laboratories, Palo Alto, CA), followed by treatment with DNaseI, extraction with phenol, and precipitation with ethanol. One microgram total RNA was amplified using GeneAmp RNA PCR Kit (Perkin-Elmer Cetus Instruments, Norwalk, CT): the RT reaction was carried out at 42 °C for 60 min with 6 ng GH8 primer and amplification was performed using primers GH7 and GH8 for 50 cycles. Each cycle consisted of 95 °C for 30 s, 60 °C for 30 s, and 70 °C for 90 s. The same amount of RNA was also amplified in the same cycles without performing the RT reaction to determine the origin of the amplified products. Ten microliters of the amplified product was electrophoresed, hybridized, and exposed to X-ray film as described above.

#### *Measurement of growth*

The age of hatchlings was counted from the day when the last individual hatched out. Hatchlings were maintained in circulating water. The water temperature was kept between 10 and 15 °C. From 40 days after hatch-out, fish were fed to satiety three times a day until 70 days after hatchling, followed by twice a day with a commercially prepared food (fry Feed Nissui, Nippon Suisan Kaisha, Ltd.). Body weight was measured five times from 39 to 140 days after hatchling. All fish of each group were weighed together at the first four measurements to minimize handling effects and weighed individually at the last measurement.

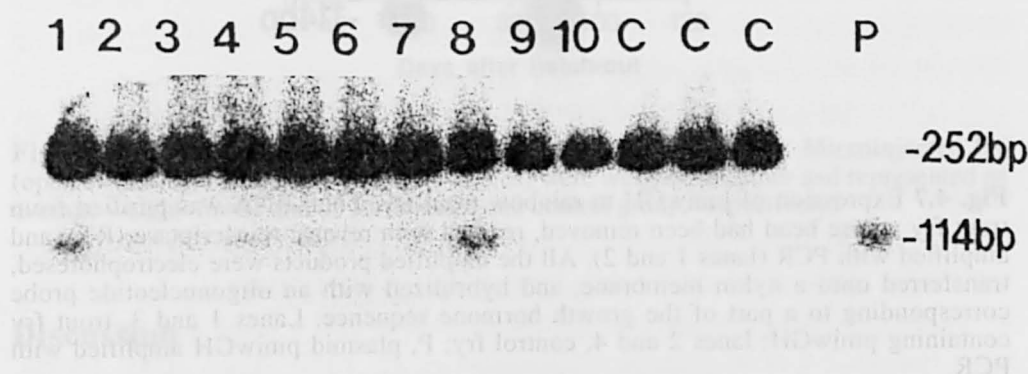
## **Results**

#### *Survival of embryos after microinjection*

About 400 unfertilized eggs were inseminated, and the fertilization rate estimated using 50 eggs was 84 %. The DNA solution was microinjected into 160 eggs and 180 were left uninjected as the control. One hundred twenty injected embryos and 130 controls hatched out 27-32 days after fertilization giving hatch-out rates of 75 % and 72 %, respectively.

### Gene transfer efficiency

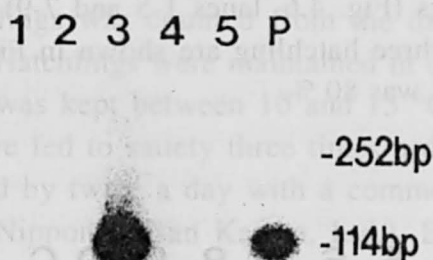
Ten injected and five control hatchlings were sampled, and DNA was extracted from the head part. The existence of introduced sequences was detected by PCR. The GH7 and GH8 primers used would amplify rtGH cDNA from the portion of +80 to +193 relative to the point of the translation initiation and generate a 114-bp fragment. In the genomic GH sequence, GH7 and GH8 are located in second and third exons, respectively, and a 138-bp intron exists between them (Agellon et al. 1988a). Therefore, these primers generate a 252-bp fragment when they amplify genomic GH gene. The foreign sequence can be distinguished easily from the genomic GH sequence of the recipient. The amplified products were transferred to a nylon filter and hybridized with an oligonucleotide probe corresponding to the portion of rtGH cDNA from +130 to +150 relative to the translation initiation site. The result of autoradiography is shown in Fig. 4.6. The 252-bp signal was detected in all hatchlings examined. The signal at 114 bp was observed in eight of 10 injected hatchlings (Fig. 4.6, lanes 1-5 and 7-9) but not in five control hatchlings (only three hatchling are shown in Fig. 4.6). Thus, the gene transfer efficiency was 80 %.



**Fig. 4.6** PCR analysis of trout fry injected with pmiwGH. DNA purified from the head part of trout fry was amplified with PCR, electrophoresed, and hybridized with an oligonucleotide probe corresponding to a part of the growth hormone sequence. Lanes 1-10, injected fry; C, uninjected fry; P, plasmid pmiwGH amplified with the same primers.

### *Expression of GH mRNA*

Total RNA was extracted from eight injected hatchlings (Nos. 1-5 and 7-9 in Fig.) that were positive for the transgene and from five controls. These RNA samples were analyzed by RT-PCR using the same primers. The result is shown in Fig. 4.7. The band at 114 bp corresponding to rtGH cDNA was detected only in hatchlings in which the transgene had been introduced (Fig. 4.7, lane 3) and not in the controls (Fig. 4.7, Lane 4). The 114-bp band was not detected when the same amount of RNA was amplified without RT reaction (Fig. 4.7, lanes 1 and 2). Thus, this band is apparently derived from mRNA, not from contaminating DNA. In addition, the band is not derived from the GH mRNA transcribed from the recipient genome because the head portion containing the pituitary gland was removed from the RNA samples. Thus, it was concluded that the rainbow trout hormone gene, encoded on the introduced plasmid, was expressed.

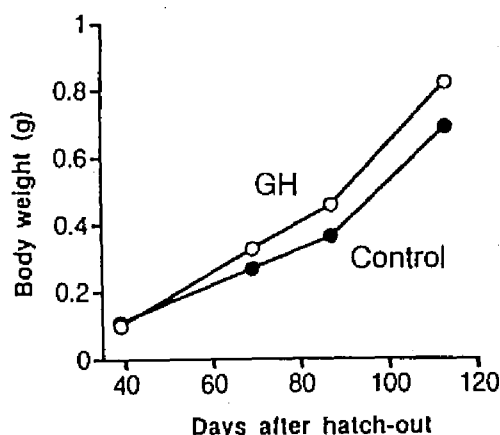


**Fig. 4.7** Expression of *pmiwGH* in rainbow trout fry. Total RNA was purified from trout fry whose head had been removed, reacted with reverse transcriptase (RT), and amplified with PCR (lanes 1 and 2). All the amplified products were electrophoresed, transferred onto a nylon membrane, and hybridized with an oligonucleotide probe corresponding to a part of the growth hormone sequence. Lanes 1 and 3, trout fry containing *pmiwGH*; lanes 2 and 4, control fry; P, plasmid *pmiwGH* amplified with PCR.

### *Growth of injected fry*

Ninety-one injected and 104 control hatchlings survived until 39 days after hatching. On this day, 13 control fry were randomly selected

and removed from the aquarium to make the number of fry in each group equal. Subsequently, the first measurement of body weight was carried out. The average weight of injected hatchlings was 90 % of that of controls (Fig. 4.8). Feeding was started from 40 days after hatch-out. From the second to fourth measurements, the average weight of injected fry was 122, 126, and 119 % of that of controls for 69, 87, and 113 days after hatch-out, respectively (Fig. 4.8) at the last measurement, i.e., 149 days, each fry was weighted individually (Table 4.1). The average weight of injected fry was 112 % that of controls, showing a significant difference ( $P < 0.05$ ). From this, it could be said that the growth of the injected group was faster than that of the uninjected controls.



**Fig. 4.8** Growth of rainbow trout fry microinjected with pmiwGH. Microinjected fry (open circles) and uninjected fry (solid circles) were weighed together and represented as average weight. (n=62 and 63 for injected and control group, respectively)

## Discussion

The rtGH was introduced into rainbow trout as a model experiment to enhance the growth of fish fry. Rainbow trout cDNA was used because the endogenous hormone was supposed to be recognized by GH receptors of the recipient better than hormones of any other species. The foreign

**Table 4.1** Weight of rainbow trout fry injected with pmiwGH 149 days after hatch-out.

	Number of fry	Average weight (g)	SD
Injected	62	1.65*	0.35
Control	63	1.47	0.43

\*Significantly different from the control ( $p < 0.05$ )

DNA was transferred by microinjection combined with glutathione treatment. This system worked well, with a high survival rate (75 % of injected eggs) and high gene transfer efficiency (80 % of hatchlings). This result may be due to the high quality of the fertilized eggs used in this experiment. The results of this study and previous studies (Yoshizaki et al. 1991, 1992) indicate that this is one of the most efficient methods for foreign gene transfer, at least in rainbow trout, although some contradictory results were shown for Atlantic salmon (Shears et al. 1992). For the analysis of DNA, PCR methods were used with a set of primers that can detect both the introduced rGH cDNA sequence and the GH gene on the recipient genome separately. A similar approach was reported by Du et al. (1992) to detect chinook salmon GH cDNA that was introduced into Atlantic salmon.

Although a number of studies have reported the introduction of foreign growth hormone gene into fish, most of them failed to detect expression. We considered two points to achieve and detect foreign gene expression in this study. One is to use the promoter that is active in the recipient fish. The promoter miw used in this study is a chimeric promoter containing a chicken  $\beta$ -actin sequence and a Rous sarcoma virus long terminal repeat sequence (Suemori et al. 1990; Kato et al. 1990), which has been shown to be active in trout embryos and fry in the previous section. In addition, the polyadenylation sequence of SV40 has been shown to function well in fish cells and fry. The second point we considered is to use a sensitive method to detect expression. Previously, gene expression was detected using immunological methods (Zhang et al.

1990; Du et al. 1992); however, these methods did not distinguish the hormone derived from transgenes from the native hormone of the recipient when homogeneous GH sequence was introduced. Rokkones et al. (1989) detected mRNA of the transgene using northern blot hybridization. As the hormones generally function at low concentrations, more sensitive detection methods are required. The RT-PCR (Rappolee et al. 1988) technique used for the detection of the transgene expression is a sensitive method for the mRNA detection (Kawasaki 1990). The expression of transgene was detected using RT-PCR combined with hybridization. It will be a powerful method to detect low-level expression of transgene in fish. The RT-PCR method used in this study is not designed for quantitative analysis. Several reports have shown that quantitative analysis is possible using RT-PCR (Gilliland et al. 1990; Wang and Mark, 1990).

The injected and control fry were weighed once before the first feeding and four times about the start of feeding. Injected fry were smaller than controls at the first measurement but became larger with subsequent measurements and a significant difference between the injected group and the control group was apparent at the fourth measurement. Thus, the effect of foreign GH expression on growth enhancement became clear after the start of feeding. Since the amount of the food consumed by each group was not determined in this study, it is not known whether the difference in weight was due to a difference in the amount of food consumed or differences in the food conversion ratio between the two groups. It has been reported that administration of recombinant GH induced aggressive feeding behavior in rainbow trout (Agellon et al. 1988b) and increased food conversion efficiency in red sea bream (Ishioka et al. 1992). Such possibilities could not be ruled out here. The average weight at four measurement after the start of feeding was 112-126 % of that of controls. The growth rate acceleration is not as high in this study as in those previously reported (Zhang et al. 1990; Du et al. 1992). However, direct comparison is difficult because the fish used in this study were quite young.

The young fry stage is a critical stage in the life cycle of fish. During this period, fish hatchlings generally complete absorption of the



yolk and start to swim and feed. This is also a period where fish are prone to infectious diseases (Wolf 1988). We have demonstrated in this study enhanced growth of young fry using a foreign plasmid including a promoter that is active in embryonic and fry stages. Results of this study indicate the possibility of accelerating the growth of fish fry at a critical stage in the life cycle using transgenic techniques.

## Chapter 5 Comprehensive discussion

Transgenic animals have mainly been used to investigate gene regulation and function in studies of genes. The regulation of genes is generally examined by introducing the 'gene of interest into the host animal and monitoring the expression. To monitor the expression, the product of the introduced gene can be detected by immunological methods as shown in Chapter 3. It is also possible to detect the expression at the RNA level as shown in chapter 4. In addition, reporter genes are useful for easy detection of expression. As reporter genes, the  $\beta$ -galactosidase gene and the chloramphenicol acetyltransferase (CAT) gene have been often used. Since both reporters were also shown to be effective in fish as described in Chapters 2 and 4, they will be utilized in transgenic researches in the future. Even deletion analysis of regulatory elements is also possible using fish embryos instead of culture cells. Microinjection into fertilized eggs of medaka is not so difficult and rather easier than transfection on culture cells once the microinjection or electroporation system was set up. In fact, Gong et al. (1991) successfully identified the functional regulatory elements of the fish antifreeze protein promoter using medaka embryos.

The function of the gene is generally studied by introducing the coding region of the gene of interest linked to suitable promoters. For this purpose, several promoters active in fish cells and embryos were identified in the chapter 2. As shown in the chapter 4, constitutive expression of transgenes became possible using constitutive promoters. Moreover, it was shown that some promoters were inducible. Using inducible promoters, expression can be stimulated at desired time. Using these promoters, it is possible to examine function of genes which may affect the development of the host. Thus, most of materials required for studying gene regulation and function have already been prepared in the process of this study.

Transgenic fish system is expected to be applicable not only to these standard experiments, but also to special experimental methods e. g., the "enhancer trap" and the "insertional mutagenesis", both of which are known to be excellent methods to isolate the genes regulating the

development. The former is a method to detect tissue- and stage -specific enhancers by introducing reporter genes linked to a weak promoter. By determining the position of the insertion of transgene in the genome of individuals revealing strong transgene expression in the target tissue, tissue specific enhancers can be isolated. The latter is to isolate genes which participate in morphogenesis by introducing a foreign gene. Morphogenic genes can be identified by determining the sequence around the point of the transgene insertion into the genome of the mutant. These methods have been used on mice but significant effort is required to treat a large number of transgenic mice. Fish which spawn large number of eggs is supposed to be more suitable for such experiments.

In the field of medical sciences, transgenic mice have been used as disease models. It is impossible to replace mice by fish entirely because most of human diseases are not common with fish. However, fish is a member of vertebrates and it seems that some of diseases including cancer and some genetic diseases can be reproducible in fish. Concerning cancer, an excellent model is available in tropical fish, platy and swordtail. Hybrids of these species develop melanoma reproducibly (Anders and Anders, 1978; Ozato and Wakamatsu 1983; Schwab, 1987). Such system combined with transgenic technique may bring us valuable information on tumorigenesis. Fish is also a potent model to study neural diseases because neural development in vertebrates is being studied actively using zebrafish (Kimmel, 1989). In fact, efforts to produce mutant strains in zebrafish to study neural development has already started in several countries. In relation to these projects, transgenic system in zebrafish is also being actively studied (Stuart et al. 1988, 1990; Culp et al. 1991; Westerfield et al. 1992). If disease models were established in fish species such as medaka and zebrafish, they would be valuable because such species are easy to maintain under laboratory conditions and not so expensive. In addition, fish is easier to be enclosed than mice because fish cannot escape from aquarium to environment without enough water.

Aquaculture of fish is thought to be indispensable to maintain fish resources. However, the history of fish culture is shorter than that of plants and farm animals because fisheries has been dependent upon only

natural resources for a long time. At present, few special strains for aquaculture is available. Thus breeding of economically important species is required. However, breeding through traditional methods takes too much time because generation time of most of fish species is more than 2 years. One possible technique to improve fish in relatively short time is the chromosome sets manipulation. Chromosome sets manipulation is possible by UV- or  $\gamma$ -ray irradiation and applying hydrostatic pressure or heat shock to gamete. Using this technique, genetically uniform population can be produced within several generations. However, chromosomes were manipulated as a haploid set and it is impossible to manipulate a single trait using this technique.

The other technique expected to become a novel method for fish breeding is foreign gene transfer examined in this study. The potential of the transgenic technique as a method for breeding was partly shown in this study. Growth of rainbow trout fry was successfully accelerated by introducing foreign growth hormone cDNA (chapter 4). It is apparent that fish phenotype can be modified by foreign gene transfer. However, many hurdles are remaining before practical breeding of fish by foreign gene transfer. The most serious problem is that few genes are available to introduce into fish at present because molecular-biological studies in fish started only recently. Only growth hormone genes and the antifreeze protein gene have been introduced for improvement of fish. The former is partially successful (Zhang et al. 1989; Du et al. 1992) but the latter has not succeeded yet until now (Hew et al. 1992). More information about fish genes should be accumulated for more progress. Transgenic fish researches have just been started. Further progress depends on the progress of molecular-biological studies of fish. Molecular-biological studies in fish will be accelerated using the transgenic techniques established in this study.

## Chapter 6 Summary

The technique for transferring foreign genes into animals is a powerful method to study gene regulation and function of genes *in vivo*. Successful foreign gene transfer has been reported in various species including nematodes, *Drosophila*, sea urchin, amphibians, mice and farm animals. The application of foreign gene transfer technique to fish began about 5 years later after the first report in mice in spite of the importance of fish not only as experimental animals but also as food resources. This study has been performed to establish the system for foreign gene transfer which enable to achieve introduction, expression and germline transmission of transgenes in fish. Another objective of this study is to evaluate the potential of the foreign gene transfer technique as a tool for basic studies of genes as well as genetic engineering of fish.

In Chapter 1, three different methods for foreign gene transfer into fish were described using medaka (*Oryzias latipes*) as a model animal. In Section 1.1, microinjection into oocyte nuclei was examined. Foreign genes were successfully introduced by injecting foreign DNA into oocytes collected 14–8 h before ovulation and the most appropriate stage was 8 h before ovulation (Section 1.1). By adding pregnant mare gonadotropin to the culture medium for oocytes, it became possible to introduce oocytes collected 20–16 h before ovulation. In Section 1.2, microinjection into fertilized eggs was examined (Section 1.2). It was shown that foreign genes can be introduced by injecting foreign DNA into the cytoplasm of fertilized eggs at the 1-cell stage. In the section 1.3, foreign gene transfer by electroporation was examined. It was shown that foreign genes can be transferred by immersing fertilized eggs in DNA solution and applying electric pulses to them. It was also shown that introduced genes are inherited to F1 and F2 generations. Efficiencies of foreign gene transfer of the three methods were compared by introducing the same plasmid pMV-GH. Among the three methods, electroporation was most simple but its gene transfer efficiency was the lowest. Microinjection into gene oocytes is the best with regard to the gene transfer efficiency although it involves complicated procedures and applicable only to limited species. Microinjection into fertilized eggs is less difficult than into oocytes but is

supposed to be obstructed by the chorion in some species. Thus, it seemed important to choose a method according to the purpose of the experiment and the species used.

Activities of various promoters derived from fish as well as other animals in cultured fish cells and fish embryos were examined to achieve transgene expression as described in Chapter 2. In the cell line RTL-4, several viral elements such as pSV2 vector and the long terminal repeat of Rous sarcoma virus, and a chicken chimeric promoter miw were constitutively active. The *Drosophila* Hsp70 promoter and metallothionein promoters derived from rainbow trout and mice were active when cells were stimulated by heat shock and metal-treatment, respectively (Section 2.1). *In vivo* activities of pSV2, miw and metallothionein promoters were also examined using medaka embryos (Section 2.2). As the result, it was shown that pSV2 and miw are also constitutively active and metallothionein promoters are active after exposure to zinc. These constitutive and inducible promoters were used to express transgenes in transgenic fish; the basic techniques to achieve introduction, expression and germ-line transmission of transgenes were thus established.

Expression of chicken  $\delta$ -crystallin gene during embryonic development was examined using a microinjection system in medaka as described in Chapter 3. In the lens, the expression was detected only in the lens-formation stage. In non-lens tissues, expression was barely detectable only in mesodermal tissues in early stages but became detectable in greater varieties of tissues including ectodermal, mesodermal and endodermal tissues. These result suggest the dual regulation of  $\delta$ -crystallin gene expression by lens-specific and non-specific regulatory elements. It was shown that the transgenic system established in this study is useful for *in vivo* studies of genes.

In Chapter 4, potential of transgenic techniques as a method to improve economically important fish species was examined using rainbow trout as a model. Methods for introducing foreign genes into rainbow trout were examined (Section 4.1). It was found that foreign genes can be transferred by microinjection into fertilized eggs after the glutathione treatment. Activity of miw, one of the most active promoters in medaka embryos, was examined in rainbow trout (Section 4.2). It was shown that

miw is active in trout embryos and fry. In 7-day-old embryos, the expression was detected in various types of tissues. In hatchlings, the expression was observed in the yolk sac. Since it was found that miw is active in rainbow trout, the rainbow trout growth hormone cDNA was linked to miw and microinjected into rainbow trout eggs (Section 4.3). The expression in hatchlings was detected by reverse transcription-polymerase chain reaction. Injected and control fry were weighed from 39 to 149 days after hatch-out and it was shown that the growth of injected fry was significantly faster than that of the control group ( $p < 0.05$ ). Results of this study indicate the potential of transgenic techniques as methods for genetic engineering of fish.

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Inoue, K., Ozato, K., Kondoh, H., Iwamatsu, T., Wakamatsu, T., Fujita, T. and Okada, T. S. (1989) Stage-dependent expression of the chicken  $\delta$ -crystallin gene in transgenic fish embryos. *Cell Differ. Dev.* 27: 57–68.

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